Published in final edited form as:

J Immunol Methods. 2018 April ; 455: 80–87. doi:10.1016/j.jim.2018.02.002.

A sensitive and selective ELISA methodology quantifies a demyelination marker in experimental and clinical samples

Albert G. Remacle¹, Jennifer Dolkas^{2,4}, Mila Angert^{2,4}, Swathi K. Hullugundi^{2,4}, Andrei V. Chernov¹, R. Carter W. Jones III^{2,3,4}, Veronica I. Shubayev^{2,4,*}, and Alex Y. Strongin^{1,*,#}

¹Infectious and Inflammatory Disease Center/Cancer Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037

²Department of Anesthesiology, University of California, San Diego, La Jolla, CA 92093

³Center for Pain Medicine, University of California, San Diego, La Jolla, CA 92093

⁴VA San Diego Healthcare System, La Jolla, CA 92037

Abstract

Sciatic nerve chronic constriction injury (CCI) in rodents produces nerve demyelination *via* proteolysis of myelin basic protein (MBP), the major component of myelin sheath. Proteolysis releases the cryptic MBP epitope, a demyelination marker, which is hidden in the native MBP fold. It has never been established if the proteolytic release of this cryptic MBP autoantigen stimulates the post-injury increase in the respective circulating autoantibodies. To measure these autoantibodies, we developed the ELISA that employed the cryptic 84-104 MBP sequence (MBP84-104) as bait. This allowed us, for the first time, to quantify the circulating anti-MBP84-104 autoantibodies in rat serum post-CCI. The circulating IgM (but not IgG) autoantibodies were detectable as soon as day 7 post-CCI. The IgM autoantibody level continually increased between days 7 and 28 post-injury. Using the rat serum samples, we established that the ELISA intra-assay (precision) and inter-assay (repeatability) variability parameters were 2.87% and 4.58%, respectively. We also demonstrated the ELISA specificity by recording the autoantibodies to the liberated MBP84-104 epitope alone, but not to intact MBP in which the 84-104 region is hidden. Because the 84-104 sequence is conserved among mammals, we tested if the ELISA was applicable to detect demyelination and quantify the respective autoantibodies in humans. Our limited pilot study that involved 16 female multiple sclerosis and fibromyalgia syndrome patients demonstrated that the ELISA was efficient in measuring both the circulating IgG- and IgM-type autoantibodies in patients exhibiting demyelination. We believe that the ELISA measurements of the circulating autoantibodies against the pathogenic MBP84-104 peptide may facilitate the identification of demyelination in both experimental and clinical settings. In clinic,

*Corresponding authors: Alex Y. Strongin, Sanford Burnham Prebys Medical Discovery Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA; T 858.795.5271; strongin@sbpdiscovery.org, and Veronica I. Shubayev, Department of Anesthesiology, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0629, USA; T. 858.534.5278; vshubayev@ucsd.edu.

#Equal senior co-authorship

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest. The authors report no conflict of interest.

these measurements may assist neurologists to recognize patients with painful neuropathy and demyelinating diseases, and as a result, to personalize their treatment regimens.

Keywords

ELISA; myelin; demyelination; myelin basic protein; multiple sclerosis; fibromyalgia; neuroimmune disease; nerve injury; experimental animals

INTRODUCTION

Myelin basic protein (MBP) is a major component of the myelin shield in the central and peripheral nervous systems (CNS/PNS). Because MBP is an intrinsically unstructured and abundant cationic protein, MBP and its fragments exhibit a multitude of physiological functions including, but not limited to, interactions with anionic lipids and actin and tubulin cytoskeletal proteins, regulation of myelin compaction and structural assembly of the axon-glia unit (1,2). Furthermore, MBP exhibits the cryptic epitopes that play a critical role in autoimmune demyelinating pathologies such as multiple sclerosis (MS) (3). These epitopes are also released in the course of PNS/CNS injury and neurodegenerative diseases.

Matrix metalloproteinase (MMP) proteolysis of MBP releases the fragments of MBP, including the cryptic epitope sequence that is hidden in the MBP native fold, into the injured nerve microenvironment (4–13). The centrally located 84–104 region of MBP [that is exemplified in our studies by a synthetic MBP84–104 peptide (ENPVVHFFKNIVTPRTPPPSQ)] is highly conserved in mammals (Figure 1a). This region includes the major immunodominant MBP84–95 T cell epitope sequence identified in patients with MS (14,15). This sequence is frequently used to induce experimental autoimmune encephalomyelitis (EAE), a murine model of human MS (14–16). Previous studies by us and others have suggested that neuropathic pain associated with both demyelinating sciatic nerve chronic constriction injury (CCI) and EAE depends on this centrally located MBP epitope (17–23). A single injection of the synthetic MBP84–104 peptide, the sequence of which correlates to the MBP cryptic epitope, into the intact sciatic nerve is sufficient to stimulate robust, long-lasting, T cell-dependent mechanical pain hypersensitivity and neuroinflammation (9,17,22–24). Because of the absence of a reliable methodology, it remained unknown whether an increase in the autoantibodies follows the release of the cryptic MBP84–104 epitope after painful peripheral mononeuropathy (25,26). Currently, there are no simple serum tests for detecting biomarkers of demyelination, and these tests remain an unmet clinical need, especially in MS (27). For example, in MS diagnosis the current blood tests, including ELISAs, help to rule out lookalike diseases that share some similar symptoms with MS, but which require distinct treatment regimens relative to MS (27–29).

To specifically quantify the serum concentrations of the autoantibodies against the cryptic 84–104 MBP epitope we developed a streamlined ELISA methodology. This ELISA employed the immobilized synthetic MBP84–104 peptide as bait for capturing the circulating anti-MBP84–104 IgG or IgM autoantibodies from the sample, and then the horseradish peroxidase (HRP)-labeled species-specific IgG or IgM antibodies for the

detection of the captured complex. The scrambled peptide (SCR) was used as a control. Our multiple tests demonstrated the high reproducibility of the ELISA methodology with its low intra- and inter-assay coefficient of variability (CV) values of 2.87% and 4.58%, respectively. The ELISA we developed and then optimized was used to measure the anti-MBP84-104 autoantibody levels in rats post-CCI (8,9,17). We then employed the ELISA for testing human serum samples in order to identify a cohort with demyelinating conditions (MS) relative to fibromyalgia syndrome (FMS) patients and healthy volunteers (all females).

Overall, our results suggest that seropositivity for the IgM and IgG autoantibodies against the cryptic MBP epitope represents a valuable biomarker of nerve demyelination in both experimental and clinical settings. We further believe that the ELISA tests may facilitate the rational diagnosis of painful neuropathy patients with the earlier or ongoing demyelination and help to personalize their treatment regimen and to avoid the futility of continuous and widespread use of the addictive conventional opioid analgesics in which some patients may be opioid-resistant.

MATERIALS AND METHODS

General reagents and antibodies

All reagents were purchased from Millipore and Sigma unless indicated otherwise. The HRP-conjugated goat anti-rat IgM (#3020-05) and goat anti-rat IgG (#112-035-175) was from Southern Biotech and Jackson ImmunoResearch, respectively. A 3,3',5,5'-tetramethylbenzidine substrate (TMB/E) and IgG & protease-free BSA (a 30% solution) were from Surmodics and US Biological, respectively.

MBP and peptides

The synthetic wild-type (WT; ENPVVHFFKNIVTPRTPPPSQ) and scrambled (SCR; EFPHIKVTVVTPRNGFPNSPP) MBP84-104 peptides (97–99% purity) were synthesized by GenScript and protected from exoprotease degradation by N-biotinylation and C-terminal amidation, respectively. Peptides are numbered according to the human MBP sequence (GenBank #AAH08749). Human intact MBP (an 18.5 kDa isoform) was from Meridian Life Science.

Animal model and serum sample collection

Eight adult female Sprague-Dawley rats (200–225 g) were obtained from Envigo Labs and housed in a temperature-controlled room (22°C) on a 12-h light/dark cycle with free access to food and water. Animals were anesthetized with 4% isoflurane in oxygen (Aerrane; Baxter) and then the common sciatic nerve was exposed unilaterally at the mid-thigh level. The nerve received three loosely constrictive chromic gut ligatures to produce CCI (9,17). At day 0 (prior injury) and days 7, 14 and 28 post-CCI blood samples (1–2 ml, each) were collected without anticoagulant from the same cohort of rats. Blood samples were allowed to clot for 30 min at ambient temperature, centrifuged ($2,000 \times g$; 10 min; 4°C) and the supernatant serum was stored at –80°C. All animal procedures were performed according to the PHS Policy on Humane Care and Use of Laboratory Animals with the experimental protocol approved by the Institutional Animal Care and Use Committee at the VA San Diego

Healthcare System, and complied with ethical guidelines of the International Association for the Study of Pain.

ELISA to detect the anti-MBP84-104 epitope IgG and IgM autoantibodies in serum samples

The wells of a 96-well Maxisorp ELISA plate (Thermo Scientific) were coated for 18 h at 4°C with ExtrAvidin (3 µg/ml in 0.125 ml 15 mM bicarbonate buffer, pH 9.6). Non-specific binding was blocked for 1 h at 37°C using 1% IgG & protease-free BSA (0.4 ml) in 50 mM Tris-HCl buffer, pH 7.8, containing 1 M NaCl and 0.1% Tween-20 (TBS/T). After six washes (5 min, each; 500-700 rpm) in TBS/T at ambient temperature, the biotin-labeled WT and SCR MBP84-104 peptides (5 µg/ml in 0.1 ml TBS/T-1% BSA, each) were added to the wells. Incubation was continued at 4°C for an additional 16-18 h. The follow-on procedures were carried out at ambient temperature and gentle agitation (500-700 rpm). After six washes (5 min, each) in TBS/T, rat or human serum samples (diluted 1:50-150 in 0.1 ml TBS/T-1% BSA) were allowed to bind to the peptide-coated wells for 3 h. Following extensive washes in TBS/T (5 min, each), the secondary HRP-conjugated species-specific IgG or IgM antibodies (diluted 1:10,000 in 0.1 ml TBS/T-1% BSA, each) were added to the wells for 1 h. After extensive washes in TBS/T (5 min, each), and then with H₂O, the TMB/E substrate (0.1 ml) was added to the wells for 15-60 min. The reaction was stopped by adding 1 M H₂SO₄ (0.1 ml). The resulting A₄₅₀ value was measured using a plate reader. Data are means ± standard deviation (SD) from at least 3 individual experiments performed in two-four replicates.

Cuff-off and threshold value determination for the ELISA

The cut-off value for the ELISA measurements with the WT and SCR MBP84-104 peptides were determined using eight normal serum samples from female intact, healthy animals analyzed in four replicates. To reach insignificant experimental fluctuations that may occur in the course of the ELISA procedure, all serum samples (1:50 dilution, each) were used in the same ELISA plate to determine the A₄₅₀ values for the WT and SCR peptides each. The threshold values for the WT and SCR were then calculated as 3 SD above the mean of the samples.

The ELISA assay specificity

The specificity of the ELISA was evaluated using serum samples collected from rats prior to CCI and at days 7, 14 and 28 post-CCI (n=4/group, each) and the full-length intact MBP as bait. The wells were coated with MBP (3 µg/ml in 0.125 ml 15 mM bicarbonate buffer, pH 9.6). The wells coated with BSA (3 µg/ml) served as a control. TBS/T-1% BSA (0.4 ml) was used to block the non-specific binding. The plates were incubated for 3 h at ambient temperature with the serum samples (diluted 1:50 in 0.1 ml TBS/T-1% BSA). The follow-on steps were as described above.

The ELISA assay reproducibility

The precision and repeatability of the ELISA were evaluated using serum samples collected from rats prior to CCI and at days 7, 14 and 28 post-CCI (n=4/group, each). The samples were analyzed for the IgM autoantibodies to determine the A₄₅₀ values against the WT and

SCR baits, and then to calculate the intra- and inter-assay CV. To calculate the intra-assay CV, two replicates of 48 serum samples (1:50–150 dilution) were tested in the same plate using the WT and SCR baits. To determine the inter-assay CV, four replicates of four serum samples with the different anti-MBP84-104 autoantibody levels (threshold, low, intermediate and high levels at day 0, 7, 14 and 28, respectively) were repeatedly and independently tested in the four individual plates against the WT and SCR baits.

Human serum samples

Serum samples were obtained from sixteen patients who were recruited from two different studies, both approved by the Institutional Review Board at UCSD, where the studies were conducted. Six patients with MS and two healthy volunteers (all females) were recruited from the Multiple Sclerosis Clinic, the Department of Neurology, UCSD, and eight female patients with FMS were enrolled from the primary care and Center for Pain Medicine clinics at UCSD. None of the subjects showed any clinical sign of active infection at the time of sample collection. Serum samples were handled using standard procedures and then stored at -80°C until use.

RESULTS

ELISA concept

The cryptic immunodominant epitope is hidden in intact MBP. In the course of demyelination and limited MMP proteolysis of MBP, this epitope is released and could be recognized by the immune system. The identification and then the quantification of the released MBP epitope in the serum is exceedingly difficult. Conversely, based on the studies by us and others (8,9,17,22,24,30–33), we speculated that an elevated level of the circulating antibodies to this epitope would be a reliable marker of demyelinating pathologies. However, whether the raise in the autoantibodies follows the release of the immunodominant MBP epitope in demyelinating neuropathy caused by traumatic nerve injury was not known.

To measure the level of these autoantibodies in the serum, we developed a robust, streamlined ELISA methodology to quantify both circulating IgM- and IgG-type autoantibodies against the cryptic immunodominant 84-104 MBP sequence region (exemplified in our study by a synthetic MBP84-104 peptide). The ELISA employed the biotin-labeled synthetic WT and SCR (control) MBP84-104 peptides as bait. The peptides were immobilized on the ExtrAvidin-coated wells of a 96-well plate. The baits were used to capture the circulating anti-MBP84-104 autoantibodies from the serum. The HRP-labeled species-specific IgG or IgM antibodies were then used to detect the captured complex (Figure 1a). Because demyelinating autoimmune diseases are prevalent in females (34–36), we used female subjects alone in our study.

ELISA optimization

The ELISA was designed and then optimized using serum samples obtained from the control and experimental female rats. The latter exhibited experimental mononeuropathy caused by CCI of sciatic nerve. Serum samples were collected prior to CCI and then at days 7, 14 and 28 post-CCI, and then used for the ELISA optimization.

Multiple tests were performed to optimize the ELISA performance, reproducibility and sensitivity. As a result, we established the optimal concentration of ExtraAvidin for plate coating (3 µg/ml), the requirements for the use of IgG & protease-free 1% BSA for blocking non-specific binding, the optimal concentration of the biotin-labeled MBP84-104 peptides for their immobilization on the ExtraAvidin-coated plastic (5 µg/ml), the optimal dilution range (1:50) of rat serum samples for the subsequent tests, the optimal dilution (1:10,000) of the secondary HRP-conjugated species-specific IgG or IgM antibodies, the optimal buffer composition used for sample incubation and plate washing (Tris-buffered saline containing 1 M NaCl and 0.1% Tween-20) and multiple additional experimental parameters.

Following the ELISA protocol optimization, we determined the cut-off value of the methodology we developed. For these purposes, we, first, used serum samples of eight female intact rats and the WT and SCR peptides as baits. To avoid plate-to-plate variations, the four replicates of each sample were analyzed for the IgG and IgM levels in the same 96-well plate. In these samples, the A_{450} values for the IgM autoantibodies were recorded for the SCR and WT peptides [$A_{450}=0.194\pm0.015$, range, 0.165–0.213 and $A_{450}=0.235\pm0.021$, range, 0.198–0.269, respectively] (Table 1 and Figure 2a, day 0). The threshold value that differentiates the positive and negative samples was calculated as 3 standard deviations above the mean of the samples. Thus, the threshold value for the IgM with the SCR peptide equaled $0.198+3\times0.015=0.237$. Any sample with $A_{450} \geq 0.237$ was then considered positive (Table 1). Similarly, we calculated the ELISA IgM threshold value for the WT peptide (0.298), and then any sample with $A_{450} \geq 0.298$ was considered positive. In contrast, in the intact rat serum samples the mean A_{450} values for the IgG were exceedingly low for both WT and SCR peptides ($A_{450}=0.022\pm0.002$ and $A_{450}=0.021\pm0.003$, respectively). The calculated threshold values for the WT and SCR peptides were 0.028 and 0.030, respectively (Figure 2a, day 0).

We next measured the levels of circulating anti-MBP84-104 IgG and IgM autoantibodies in the intact rat serum samples (day 0) and also in the samples obtained at days 7, 14 and 28 post-CCI. The IgM autoantibodies against the WT peptide were readily detected in the serum at day 7 (an average $A_{450}=0.065$ above the threshold). The IgM levels continued to increase at days 14 and 28 with an average $A_{450}=0.134$ and $A_{450}=0.323$ above the threshold, respectively. In turn, the levels of the IgM autoantibodies against the SCR peptide did not change significantly over time and remained similar to the threshold value. We then calculated the levels of the specific circulating IgM autoantibodies against the WT epitope as the difference in the average A_{450} value between the WT and the SCR peptides. Thus, relative to the threshold level in the intact rats, the specific anti-MBP84-104 IgM autoantibody pool increased 2.4-, 3.5- and 6.8-fold at days 7, 14 and 28 post-CCI, respectively (Figure 2a). Contrary to the seropositivity for the IgM autoantibodies, seronegativity was recorded for the anti-MBP84-104 IgG autoantibodies, likely because of the short-time nature of our experiments and the insufficient time to raise the IgG pool against the newly exposed immunodominant antigen (Figure 2a). Overall, our data support our suggestion that MBP is proteolysed shortly after nerve injury, and then its cryptic epitope is released, inducing as a result, the anti-epitope IgM autoantibodies, the first antibodies produced by the immune system exposed to a novel antigen. We have to emphasize that the ELISA methodology represents a novel diagnostic tool to detect the

circulating autoantibodies against the cryptic MBP peptide associated with demyelination. It remains undetermined if a correlation between the autoantibody levels and the pain status exists.

ELISA reproducibility and specificity

To assess the reproducibility and specificity of the ELISA, we used the rat serum samples with the increasing level of the anti-MBP84-104 IgM autoantibody (at days 0, 7, 14 and 28 post-CCI). To establish the reproducibility of the ELISA, we determined the intra-assay CV (precision) and inter-assay CV (repeatability). Thus, using the WT and SCR baits, we measured the IgM levels in 48 serum samples (two replicates each) in the same plate to calculate the intra-assay CV (2.87%; a 0.16% to 6.68% range) (Table 2). To establish the inter-assay CV for the IgM antibodies, we analyzed the same four serum samples (four replicates each) on four different plates using both the WT and SCR baits. The inter-assay CV was then calculated to equal 4.58% (a 2.96% to 7.20% range) (Table 3). Intra- and inter-assay CV below 10% are normally considered acceptable for diagnostic ELISA tests (37,38) indicating the compliance of the ELISA we developed with the generally accepted standards.

To evaluate the specificity of the ELISA, we used the full-length MBP in which the epitope is hidden. Thus we analyzed the rat serum samples (collected prior injury and at days 7, 14 and 28 post-CCI) using intact MBP, instead of the WT or SCR peptides, as bait. There was no significant difference between full-length MBP and a BSA control for neither IgM nor IgG autoantibodies (Figure 2b). Based on these results, we concluded that the level of the autoantibodies against full-length MBP was insignificant, if any, in the rat serum post-CCI. Conversely, these data suggest again that native MBP is proteolyzed after nerve injury and that the released cryptic epitope, rather than the full-length MBP protein, is recognized as a novel antigen that engages a response by the immune system resulting in the specific pool of the anti-MBP84-104 epitope autoantibodies. Overall, the ELISA methodology we established allowed us to assess, both quantitatively and in a precise and repeatable fashion, the level of the circulating specific IgM autoantibodies against the cryptic MBP84-104 epitope fragment released post-CCI in the rat serum.

Evaluation of human samples

Because the MBP84-104 sequence is conserved in rodents and humans (Figure 1), we next determined if the ELISA was applicable to human serum samples. For these purposes, we performed a limited study that included sixteen serum samples. These samples were obtained from six patients with MS (a demyelinating disease characterized by the autoantibody presence to this immunodominant MBP epitope (14–16,25)), from eight patients with FMS (a presumably, non-demyelinating disease in which the presence of autoantibodies to this MBP epitope was not known) and two healthy, control, volunteers (all females).

One MS patient who received Copaxone, a drug known to interfere with immune system(39), exhibited confusing measurements and, as a result, was excluded from our analysis. Our ELISA measurements showed that in the serum from MS patients, the anti-

MBP84-104 IgG autoantibodies were readily detectable (average $A_{450}=0.417$, a 0.147–0.854 range), whereas the healthy volunteers were seronegative (average $A_{450}=0.012$) (Figure 3a). Relative to healthy control, the circulating IgG autoantibody level was elevated 12.6–73.3-fold (average=35.8-fold). Relative to healthy volunteers, a less noticeable, 4.4–12.1-fold increase (average=6.5-fold) in the IgM autoantibodies was also recorded in MS patients. Because MS is a long-term demyelinating disease with episodic relapses, the predominant presence of the IgG antibodies against the cryptic epitope is not surprising.

In contrast with MS, demyelination is not common in FMS. Some studies indicate that FMS does not involve nerve demyelination, whereas a few others claim otherwise and, as a result, there is no consensus as yet (40–44). To detect if the autoantibodies to MBP84-104 were present in FMS, we analyzed the serum samples from FMS patients. Our measurements recorded that, in contrast to MS, FMS patients exhibited a significantly lower level of the circulating IgG autoantibodies. On average, the anti-MBP84-104 IgG level was ~13-fold lower in FMS patients than in MS patients (Figure 3b). Intriguingly, four from eight FMS patients exhibited the measurable, albeit very low, IgG seropositivity that was ~5.5-fold higher relative to the healthy volunteers, while the other four patients were seronegative. The level of the specific IgM autoantibodies was exceedingly low or even below detection level in all FMS samples.

In conclusion, our current study demonstrated as a proof-of-concept that the ELISA methodology we developed delivers the reproducible measurements of the circulating IgG and IgM autoantibodies against the cryptic MBP epitope that is released as a result of nerve demyelination in both experimental and clinical settings.

DISCUSSION

MBP is an established autoantigen in patients with autoimmune demyelinating MS and the respective experimental EAE models (3,14,16,45). In rodent models of traumatic peripheral nerve injury, including CCI, the cryptic central MBP epitope is released *via* MMP proteolysis (8,9,17). It is important to emphasize that the MBP84-104 sequence region is conserved in humans and rodents. This cryptic central MBP epitope has been implicated in neuropathic pain associated with both CCI and EAE (17–21). Further, a single bolus, adjuvant-free injection of the MBP84-104 epitope peptide into intact sciatic nerve is sufficient to produce robust mechanical pain hypersensitivity in female rats lasting for weeks, in the absence of overt neuropathology or widespread neuroinflammation (9,23).

Whereas circulating autoantibodies against the algescic MBP epitope are believed to contribute to MS and EAE, whether their levels are elevated in painful peripheral demyelinating neuropathy was not known. To generate a tool that may help to answer these questions, we developed the robust, sensitive and reproducible ELISA methodology. This ELISA measures the circulating anti-MBP84-104 cryptic epitope autoantibodies in serum samples. Using the ELISA we developed, we demonstrated, for the first time, that the level of the IgM, but not IgG, autoantibodies continuously increased in female rats after nerve injury. The upregulation of the IgM-type antibodies, the first antibodies isotype B cells produce in response to a novel foreign antigen exposure (28), may relate to the short time-

frame of our neurotrauma experiments. It is well established that the avidity (accumulated strength of multiples affinities) of the pentameric IgM antibodies with 10 antigen-binding sites are superior relative to monomeric IgG that only contains 2 antigen binding sites.

To provide, ultimately, an additional tool for diagnosis of demyelinating pathologies, we tested if the ELISA we developed and validated in rats is applicable for the analysis of human serum. We determine that the ELISA readily discriminated MS patients from healthy volunteers and recorded the high level of the anti-MBP84-104 IgG autoantibodies in MS. In turn, the autoantibody level was low or nonexistent in FMS patients, a disease in which widespread nerve demyelination is uncommon (46). Neuropathic pain featuring allodynia, lancinating and burning pain is common in MS (47). Females constitute ~80% of patients with autoimmune conditions and are more common sufferers of MS, FMS and chronic pain in general (34–36,48-51). The MBP84-104 ELISA we developed is applicable for analyzing blood samples. This ELISA employs widely available and inexpensive reagents, and provides rapid measurements with a diagnostic value for demyelinating diseases, a capacity that other current blood tests are lacking.

Overall, we believe that the cryptic immunodominant 84-104 epitope of MBP represents a reliable nerve demyelination marker and that the ELISA we developed is a promising test to facilitate the rational diagnosis of demyelinating pathologies and to supplement the currently existing diagnostic protocols in neuropathy. The present study provides the first evidence for the presence of the circulating autoantibodies against the cryptic MBP epitope in the model of painful traumatic nerve injury. This is consistent with the emerging role of neural antigens in nociceptive pathways (25,26,52–56). Furthermore, because neuropathic pain in demyelinating pathologies, including MS, is poorly responsive to opioid analgesics (29,57), the use of this ELISA may also help to avoid opioid overuse.

Acknowledgments

This work was supported by NIH R01 DE022757 (to VIS and AYS), the Department of Veterans Affairs 5I01BX000638 (to VIS), National Multiple Sclerosis Society PR-1503-03449 (to VIS and AYS) and the UCSD Clinical and Translational Science Program UL1TR001442 (to AGR) grants. We are grateful to Lucinda Martin, Katie Lam, Drs. Sabrina Oukil, Laura Muehl, Rip Kinkel and Tobias Moeller-Bertram for their help in collecting human serum samples.

Abbreviations

CCI	chronic constriction injury
FMS	fibromyalgia syndrome
HRP	horseradish peroxidase
MBP	myelin basic protein
WT and SCR MBP84-104	the wild-type and scrambled 84-104 peptide of MBP
MS	multiple sclerosis

TBS/T

50 mM Tris-HCl buffer, pH 7.8, containing 1 M NaCl and 0.1% Tween-20

References

1. Boggs JM. Myelin basic protein: a multifunctional protein. *Cell Mol Life Sci.* 2006; 63:1945–1961. [PubMed: 16794783]
2. Harauz G, Boggs JM. Myelin management by the 18.5-kDa and 21.5-kDa classic myelin basic protein isoforms. *J Neurochem.* 2013; 125:334–361. [PubMed: 23398367]
3. Kadlubowski M, Hughes RA. Identification of the neuritogen for experimental allergic neuritis. *Nature.* 1979; 277:140–141. [PubMed: 310522]
4. Chandler S, Coates R, Gearing A, Lury J, Wells G, Bone E. Matrix metalloproteinases degrade myelin basic protein. *Neurosci Lett.* 1995; 201:223–226. [PubMed: 8786845]
5. Chandler S, Cossins J, Lury J, Wells G. Macrophage metalloelastase degrades matrix and myelin proteins and processes a tumour necrosis factor- α fusion protein. *Biochem Biophys Res Commun.* 1996; 228:421–429. [PubMed: 8920930]
6. D'Souza CA, Moscarello MA. Differences in susceptibility of MBP charge isomers to digestion by stromelysin-1 (MMP-3) and release of an immunodominant epitope. *Neurochem Res.* 2006; 31:1045–1054. [PubMed: 16871440]
7. Gijbels K, Proost P, Masure S, Carton H, Billiau A, Opdenakker G. Gelatinase B is present in the cerebrospinal fluid during experimental autoimmune encephalomyelitis and cleaves myelin basic protein. *J Neurosci Res.* 1993; 36:432–440. [PubMed: 7505841]
8. Kim Y, Remacle AG, Chernov AV, Liu H, Shubayev I, Lai C, Dolkas J, Shiryayev SA, Golubkov VS, Mizisin AP, Strongin AY, Shubayev VI. The MMP-9/TIMP-1 axis controls the status of differentiation and function of myelin-forming Schwann cells in nerve regeneration. *PLoS One.* 2012; 7:e33664. [PubMed: 22438979]
9. Liu H, Shiryayev SA, Chernov AV, Kim Y, Shubayev I, Remacle AG, Baranovskaya S, Golubkov VS, Strongin AY, Shubayev VI. Immunodominant fragments of myelin basic protein initiate T cell-dependent pain. *J Neuroinflammation.* 2012; 9:119. [PubMed: 22676642]
10. Proost P, Van Damme J, Opdenakker G. Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein. *Biochem Biophys Res Commun.* 1993; 192:1175–1181. [PubMed: 7685161]
11. Shiryayev SA, Remacle AG, Savinov AY, Chernov AV, Cieplak P, Radichev IA, Williams R, Shiryayeva TN, Gawlik K, Postnova TI, Ratnikov BI, Eroshkin AM, Motamedchaboki K, Smith JW, Strongin AY. Inflammatory proprotein convertase-matrix metalloproteinase proteolytic pathway in antigen-presenting cells as a step to autoimmune multiple sclerosis. *J Biol Chem.* 2009; 284:30615–30626. [PubMed: 19726693]
12. Shiryayev SA, Savinov AY, Cieplak P, Ratnikov BI, Motamedchaboki K, Smith JW, Strongin AY. Matrix metalloproteinase proteolysis of the myelin basic protein isoforms is a source of immunogenic peptides in autoimmune multiple sclerosis. *PLoS One.* 2009; 4:e4952. [PubMed: 19300513]
13. Shubayev VI, Myers RR. Upregulation and interaction of TNF α and gelatinases A and B in painful peripheral nerve injury. *Brain Res.* 2000; 855:83–89. [PubMed: 10650133]
14. Jansson L, Olsson T, Hojeberg B, Holmdahl R. Chronic experimental autoimmune encephalomyelitis induced by the 89-101 myelin basic protein peptide in B10RIII (H-2r) mice. *Eur J Immunol.* 1991; 21:693–699. [PubMed: 1707002]
15. Li J, Zhao X, Hao HW, Shaw MK, Tse HY. T cells that trigger acute experimental autoimmune encephalomyelitis also mediate subsequent disease relapses and predominantly produce IL-17. *J Neuroimmunol.* 2011; 230:26–32. [PubMed: 20826011]
16. Liu Y, Teige I, Birnir B, Issazadeh-Navikas S. Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. *Nat Med.* 2006; 12:518–525. [PubMed: 16633347]
17. Hong S, Remacle AG, Shiryayev SA, Choi W, Hullugundi SK, Dolkas J, Angert M, Nishihara T, Yaksh TL, Strongin AY, Shubayev VI. Reciprocal relationship between membrane type 1 matrix

metalloproteinase and the algescic peptides of myelin basic protein contributes to chronic neuropathic pain. *Brain Behav Immun.* 2017; 60:282–292. [PubMed: 27833045]

18. Liu H, Shiryayev SA, Chernov AV, Kim Y, Shubayev I, Remacle AG, Baranovskaya S, Golubkov VS, Strongin AY, Shubayev VI. Immunodominant fragments of myelin basic protein initiate T cell-dependent pain. *J Neuroinflamm.* 2012; 9:119.
19. Perera CJ, Lees JG, Duffy SS, Makker PG, Fivelman B, Apostolopoulos V, Moalem-Taylor G. Effects of active immunisation with myelin basic protein and myelin-derived altered peptide ligand on pain hypersensitivity and neuroinflammation. *J Neuroimmunol.* 2015; 286:59–70. [PubMed: 26298325]
20. Perera CJ, Duffy SS, Lees JG, Kim CF, Cameron B, Apostolopoulos V, Moalem-Taylor G. Active immunization with myelin-derived altered peptide ligand reduces mechanical pain hypersensitivity following peripheral nerve injury. *J Neuroinflammation.* 2015; 12:28. [PubMed: 25885812]
21. Tian DH, Perera CJ, Apostolopoulos V, Moalem-Taylor G. Effects of vaccination with altered Peptide ligand on chronic pain in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. *Front Neurol.* 2013; 4:168. [PubMed: 24194728]
22. Shubayev VI, Strongin AY, Yaksh TL. Role of myelin auto-antigens in pain: a female connection. *Neural Regen Res.* 2016; 11:890–891. [PubMed: 27482203]
23. Ko JS, Eddinger KA, Angert M, Chernov AV, Dolkas J, Strongin AY, Yaksh TL, Shubayev VI. Spinal activity of interleukin 6 mediates myelin basic protein-induced allodynia. *Brain Behav Immun.* 2016; 56:378–389. [PubMed: 26970355]
24. Kobayashi H, Chattopadhyay S, Kato K, Dolkas J, Kikuchi S, Myers RR, Shubayev VI. MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage. *Mol Cell Neurosci.* 2008; 39:619–627. [PubMed: 18817874]
25. Bennett DL, Vincent A. Autoimmune pain: an emerging concept. *Neurology.* 2012; 79:1080–1081. [PubMed: 22895596]
26. Goebel A. Autoantibody pain. *Autoimmun Rev.* 2016; 15:552–557. [PubMed: 26883460]
27. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, Montalban X, O'Connor P, Sandberg-Wollheim M, Thompson AJ, Waubant E, Weinshenker B, Wolinsky JS. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol.* 2011; 69:292–302. [PubMed: 21387374]
28. Polman CH, Killestein J. Anti-myelin antibodies in multiple sclerosis: clinically useful? *J Neurol Neurosurg Psychiatry.* 2006; 77:712. [PubMed: 16705192]
29. Truini A, Galeotti F, Cruccu G. Treating pain in multiple sclerosis. *Expert Opin Pharmacother.* 2011; 12:2355–2368. [PubMed: 21916786]
30. Chattopadhyay S, Shubayev VI. MMP-9 controls Schwann cell proliferation and phenotypic remodeling via IGF-1 and ErbB receptor-mediated activation of MEK/ERK pathway. *Glia.* 2009; 57:1316–1325. [PubMed: 19229995]
31. Chernov AV, Dolkas J, Hoang K, Angert M, Srikrishna G, Vogl T, Baranovskaya S, Strongin AY, Shubayev VI. The calcium-binding proteins S100A8 and S100A9 initiate the early inflammatory program in injured peripheral nerves. *J Biol Chem.* 2015; 290:11771–11784. [PubMed: 25792748]
32. Hedegaard CJ, Chen N, Sellebjerg F, Sorensen PS, Leslie RG, Bendtzen K, Nielsen CH. Autoantibodies to myelin basic protein (MBP) in healthy individuals and in patients with multiple sclerosis: a role in regulating cytokine responses to MBP. *Immunology.* 2009; 128:e451–461. [PubMed: 19191913]
33. O'Connor KC, Chitnis T, Griffin DE, Piyasirisilp S, Bar-Or A, Khoury S, Wucherpfennig KW, Hafler DA. Myelin basic protein-reactive autoantibodies in the serum and cerebrospinal fluid of multiple sclerosis patients are characterized by low-affinity interactions. *J Neuroimmunol.* 2003; 136:140–148. [PubMed: 12620653]
34. Dooley MA, Hogan SL. Environmental epidemiology and risk factors for autoimmune disease. *Curr Opin Rheumatol.* 2003; 15:99–103. [PubMed: 12598794]
35. Gleicher N, Barad DH. Gender as risk factor for autoimmune diseases. *J Autoimmun.* 2007; 28:1–6. [PubMed: 17261360]

36. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol.* 1997; 84:223–243. [PubMed: 9281381]
37. Selman L, Henriksen ML, Brandt J, Palarasah Y, Waters A, Beales PL, Holmskov U, Jorgensen TJ, Nielsen C, Skjodt K, Hansen S. An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1). *J Immunol Methods.* 2012; 375:182–188. [PubMed: 22301270]
38. Thomsson O, Strom-Holst B, Sjunnesson Y, Bergqvist AS. Validation of an enzyme-linked immunosorbent assay developed for measuring cortisol concentration in human saliva and serum for its applicability to analyze cortisol in pig saliva. *Acta Vet Scand.* 2014; 56:55. [PubMed: 25193181]
39. Arnon R, Aharoni R. Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications. *Proc Natl Acad Sci U S A.* 2004; 101(Suppl 2): 14593–14598. [PubMed: 15371592]
40. Okifuji A, Hare BD. Management of fibromyalgia syndrome: review of evidence. *Pain Ther.* 2013; 2:87–104. [PubMed: 25135147]
41. Sluka KA, Clauw DJ. Neurobiology of fibromyalgia and chronic widespread pain. *Neuroscience.* 2016; 338:114–129. [PubMed: 27291641]
42. Walitt B, Nahin RL, Katz RS, Bergman MJ, Wolfe F. The Prevalence and Characteristics of Fibromyalgia in the 2012 National Health Interview Survey. *PLoS One.* 2015; 10:e0138024. [PubMed: 26379048]
43. Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Katz RS, Mease P, Russell AS, Russell IJ, Winfield JB, Yunus MB. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. *Arthritis Care Res (Hoboken).* 2010; 62:600–610. [PubMed: 20461783]
44. Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P, et al. The American College of Rheumatology 1990 Criteria for the Classification of Fibromyalgia. Report of the Multicenter Criteria Committee. *Arthritis Rheum.* 1990; 33:160–172. [PubMed: 2306288]
45. Harauz G, Ishiyama N, Hill CM, Bates IR, Libich DS, Fares C. Myelin basic protein-diverse conformational states of an intrinsically unstructured protein and its roles in myelin assembly and multiple sclerosis. *Micron.* 2004; 35:503–542. [PubMed: 15219899]
46. Di Franco M, Iannuccelli C, Bazzichi L, Atzeni F, Consensi A, Salaffi F, Pietropaolo M, Alessandri C, Basili S, Olivieri M, Bombardieri S, Valesini G, Sarzi-Puttini P. Misdiagnosis in fibromyalgia: a multicentre study. *Clin Exp Rheumatol.* 2011; 29:S104–108.
47. Solaro C, Messmer Uccelli M. Pharmacological management of pain in patients with multiple sclerosis. *Drugs.* 2010; 70:1245–1254. [PubMed: 20568832]
48. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL 3rd. Sex, gender, and pain: a review of recent clinical and experimental findings. *J Pain.* 2009; 10:447–485. [PubMed: 19411059]
49. Nahin RL. Estimates of pain prevalence and severity in adults: United States, 2012. *J Pain.* 2015; 16:769–780. [PubMed: 26028573]
50. Kim CF, Moalem-Taylor G. Detailed characterization of neuro-immune responses following neuropathic injury in mice. *Brain Res.* 2011; 1405:95–108. [PubMed: 21741621]
51. Zandman-Goddard G, Peeva E, Shoenfeld Y. Gender and autoimmunity. *Autoimmun Rev.* 2007; 6:366–372. [PubMed: 17537382]
52. McMahon SB, La Russa F, Bennett DL. Crosstalk between the nociceptive and immune systems in host defence and disease. *Nat Rev Neurosci.* 2015; 16:389–402. [PubMed: 26087680]
53. Wigerblad G, Bas DB, Fernandes-Cerqueira A, Krishnamurthy A, Nandakumar KS, Rogoz K, Kato J, Sandor K, Su J, Jimenez-Andrade JM, Finn A, Bersellini Farinotti A, Amara K, Lundberg K, Holmdahl R, Jakobsson PJ, Malmstrom V, Catrina AI, Klareskog L, Svensson CI. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. *Ann Rheum Dis.* 2016; 75:730–738. [PubMed: 26613766]

54. Sorkin LS, Yu AL, Junger H, Doom CM. Antibody directed against GD(2) produces mechanical allodynia, but not thermal hyperalgesia when administered systemically or intrathecally despite its dependence on capsaicin sensitive afferents. *Brain Res.* 2002; 930:67–74. [PubMed: 11879797]
55. Mifflin KA, Kerr BJ. Pain in autoimmune disorders. *J Neurosci Res.* 2017; 95:1282–1294. [PubMed: 27448322]
56. Klein CJ, Lennon VA, Aston PA, McKeon A, Pittock SJ. Chronic pain as a manifestation of potassium channel-complex autoimmunity. *Neurology.* 2012; 79:1136–1144. [PubMed: 22895588]
57. Kalman S, Osterberg A, Sorensen J, Boivie J, Bertler A. Morphine responsiveness in a group of well-defined multiple sclerosis patients: a study with i.v. morphine. *Eur J Pain.* 2002; 6:69–80. [PubMed: 11888230]

HIGHLIGHTS

- The cryptic epitope of MBP is released in demyelinating diseases
- This cryptic autoantigen stimulates the production of autoantibodies
- To quantify these antibodies, we developed a robust and sensitive ELISA methodology
- ELISA identifies demyelination pathologies in experimental and clinical settings

Figure 1.

ELISA to detect autoantibodies against the cryptic MBP peptide. **(a)** Sequence alignment of the evolutionary conserved cryptic central region of MBP. Human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), pig (*Sus scrofa*), guinea pig (*Cavia porcellus*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), cattle (*Bos taurus*), rabbit (*Oryctolagus cuniculus*), horse (*Equus caballus*) and chicken (*Gallus gallus*). Conserved residues are in bold. The cryptic MBP84-104 epitope, black square. Residues are numbered according to the human MBP sequence (GenBank #AAH08749). **(b)** Illustration of the ELISA. The biotin-labeled wild-type and scrambled MBP84-104 peptides (WT and SCR, respectively) were immobilized on the ExtrAvidin-coated wells of a 96-well plate. Serum aliquots were added to the wells to capture the circulating anti-MBP84-104 epitope IgG/IgM autoantibodies. The captured IgG/IgM autoantibodies were then detected and quantified using horseradish peroxidase (HRP)-conjugated species-specific IgG/IgM antibodies, and a HRP substrate.

Figure 2.

ELISA measurements in rat serum samples for the circulating autoantibodies against the cryptic MBP peptide. **(a)** ELISA of the anti-MBP84-104 IgG and IgM autoantibodies in the serum of rats with mononeuropathy induced by CCI. The biotin-labeled wild-type (WT, blue and green) and scrambled (SCR, red and purple) MBP84-104 peptides were immobilized on the ExtraAvidin-coated wells as antigen targets, respectively. Serum aliquots from females (n=4–8/group) collected at day 0 (prior injury) and at days 7, 14 and 28 post-CCI were allowed to bind to the peptides. The bound autoantibodies were detected using HRP-conjugated anti-rat IgM or IgG, and a TMB/E substrate. The threshold values for the ELISA for each antigen target were determined as 3 standard deviations above the mean of the rat serum samples collected prior injury (n=8). *Dotted lines*, the threshold values for the WT and SCR peptides were $A_{450} = 0.298$ and $A_{450} = 0.223$, respectively. **(b)** ELISA of the IgG and IgM autoantibodies against full-length MBP in rat serum. Intact MBP with the hidden cryptic MBP epitope (red and purple) and BSA (control; blue and green) were immobilized onto the wells as antigen targets, respectively. Serum aliquots (n=4/group) were collected and analyzed as described in **(a)**.

Figure 3.

ELISA measurements for the circulating autoantibodies against the cryptic MBP peptide in human serum. **(a)** ELISA in MS patients (demyelinating conditions). The biotin-labeled WT and SCR MBP84-104 peptides were immobilized on the ExtrAvidin-coated wells of a 96-well plate. Serum aliquots from healthy volunteers (averaged values, CTR) and MS patients (M-1, M-2, M-3, M-4 and M-5) were allowed to bind to the peptides. The bound IgG (black rectangles) and IgM (grey rectangles) antibodies were then detected using HRP-conjugated antihuman IgG or IgM, and a TMB/E substrate. *Left*, the specific A_{450} values for the WT peptide are calculated relative to the SCR peptide (A_{450} WT-SCR). *Right*, the IgG- and IgM-fold difference in the specific A_{450} WT-SCR values for the MBP84-104 peptide in MS patients relative to CTR. **(b)** ELISA in FMS patients (presumably, non-demyelinating conditions). Serum samples from eight patients (F-1, F-2, F-3, F-4, F-5, F-6, F-7 and F-8) were analyzed using the ELISA as described in **(a)**. The average A_{450} WT-SCR values for the serum of healthy volunteers (CTR) and MS patients (MS) were used for comparison. The specific A_{450} WT-SCR values for the WT peptide were calculated relative to the SCR

peptide. Black and grey rectangles, the specific IgG and IgM levels against the MBP84-104 peptide, respectively. (**a–b**) Data are means $\pm$ SD from at least three individual experiments performed in triplicate.

Table 1**The ELISA threshold values for IgM in rat serum**

Rat serum samples (1:50 dilution; n=8) from naïve animals were analyzed in four replicates using the ELISA with the immobilized WT and SCR MBP84-104 peptides. The mean value against the individual WT and SCR peptide was calculated for each animal, and then used to determine the overall mean (mean of means) and standard deviation (SD). The threshold value was calculated as 3 SD above the overall mean.

Samples	WT MBP84-104				SCR MBP84-104			
	A ₄₅₀	A ₄₅₀	A ₄₅₀	A ₄₅₀	Means	A ₄₅₀	A ₄₅₀	Means
1	0.252	0.247	0.243	0.238	0.245	0.208	0.203	0.198
2	0.220	0.224	0.230	0.234	0.227	0.210	0.199	0.201
3	0.244	0.249	0.264	0.269	0.257	0.165	0.185	0.181
4	0.229	0.246	0.242	0.258	0.244	0.192	0.196	0.193
5	0.205	0.209	0.198	0.214	0.207	0.192	0.196	0.193
6	0.241	0.243	0.248	0.250	0.245	0.190	0.192	0.182
7	0.243	0.247	0.256	0.261	0.252	0.209	0.213	0.203
8	0.204	0.189	0.207	0.200	0.200	0.197	0.199	0.197
Mean of Means					0.235	Mean of Means		
SD of Means					0.021	SD of Means		
Threshold					0.298	Threshold		

Table 2
The ELISA intra-assay coefficient of variability (CV) for the IgM measurements in rat serum

Two replicates of 48 rat serum samples (1:50–150 dilution) collected at the indicated time (0, 7, 14 and 28 post-CCI) were analysed using the ELISA with the individual WT and SCR MBP84-104 immobilized on the same plate. To determine the % CV we calculated, for each sample, the mean and standard deviation (SD) of the two A_{450} values of the replicates. The intra-assay CV (2.87%) is reported as the average of the individual % CV for the samples (a 0.16–6.68 range). Acceptable range for intra-assay CV for an ELISA was determined to be below <10%.

Samples	Peptides	Days	Dilutions	A_{450}	Means	SD	% CV
1	WT	0	50	0.241	0.252	0.015	6.14
2	WT	0	50	0.268	0.279	0.015	5.56
3	WT	0	50	0.279	0.285	0.008	2.85
4	WT	0	50	0.284	0.278	0.008	2.92
5	WT	7	50	0.364	0.375	0.016	4.18
6	WT	7	50	0.351	0.353	0.002	0.64
7	WT	7	50	0.359	0.357	0.003	0.79
8	WT	7	50	0.397	0.393	0.005	1.26
9	WT	14	50	0.421	0.423	0.003	0.64
10	WT	14	50	0.450	0.452	0.003	0.59
11	WT	14	50	0.465	0.455	0.014	3.07
12	WT	14	50	0.405	0.407	0.002	0.43
13	WT	28	50	0.623	0.625	0.001	0.16
14	WT	28	50	0.629	0.630	0.001	0.16
15	WT	28	50	0.547	0.553	0.009	1.56
16	WT	28	50	0.608	0.602	0.009	1.43
17	SCR	0	50	0.210	0.202	0.006	2.78
18	SCR	0	50	0.182	0.200	0.013	6.62
19	SCR	0	50	0.185	0.201	0.011	5.71
20	SCR	0	50	0.228	0.222	0.009	4.15
21	SCR	7	50	0.187	0.196	0.006	3.03

Samples	Peptides	Days	Dilutions	A ₄₅₀	A ₄₅₀	Means	SD	% CV
22	SCR	7	50	0.192	0.196	0.194	0.002	1.17
23	SCR	7	50	0.175	0.165	0.170	0.007	4.28
24	SCR	7	50	0.201	0.200	0.200	0.001	0.49
25	SCR	14	50	0.219	0.223	0.221	0.003	1.22
26	SCR	14	50	0.193	0.197	0.195	0.003	1.38
27	SCR	14	50	0.200	0.215	0.207	0.011	5.32
28	SCR	14	50	0.199	0.194	0.197	0.004	1.87
29	SCR	28	50	0.163	0.164	0.163	0.001	0.61
30	SCR	28	50	0.197	0.199	0.198	0.001	0.50
31	SCR	28	50	0.199	0.216	0.207	0.012	5.59
32	SCR	28	50	0.186	0.193	0.189	0.005	2.76
33	WT	0	150	0.093	0.098	0.096	0.003	3.55
34	WT	0	150	0.080	0.073	0.076	0.005	6.68
35	WT	0	150	0.107	0.102	0.104	0.003	3.25
36	WT	0	150	0.082	0.080	0.081	0.001	1.49
37	WT	7	150	0.124	0.124	0.124	0.000	0.34
38	WT	7	150	0.142	0.156	0.149	0.010	6.54
39	WT	7	150	0.124	0.129	0.126	0.004	2.80
40	WT	7	150	0.122	0.112	0.117	0.008	6.48
41	WT	14	150	0.166	0.174	0.170	0.006	3.25
42	WT	14	150	0.164	0.164	0.164	0.000	0.30
43	WT	14	150	0.169	0.177	0.173	0.006	3.30
44	WT	14	150	0.159	0.160	0.160	0.000	0.18
45	WT	28	150	0.264	0.245	0.255	0.013	5.22
46	WT	28	150	0.249	0.223	0.236	0.018	7.82
47	WT	28	150	0.242	0.262	0.252	0.015	5.78
48	WT	28	150	0.245	0.249	0.247	0.003	1.09

Intra-assay % CV 2.87

Table 3
The ELISA inter-assay CV for the IgM measurements in rat serum samples with the threshold, low, intermediate and high levels of the anti-MBP84-104 autoantibodies

Four replicates of four rat serum samples (1:50 dilution) with the threshold, low, medium and high levels of the IgM autoantibodies were each analysed on four individual plates, using the individual WT and SCR MBP84-104 immobilized on the same plate. For each plate and each IgM autoantibody level, we determined the A_{450} WT-SCR value, using the average A_{450} values of the replicates against the WT and SCR peptides. To determine the % CV for the four individual IgM autoantibodies levels, we calculated the mean and standard deviation (SD) of the four A_{450} WT-SCR mean values recorded on each of the individual plates. The inter-assay CV (4.58%) is reported as the average of the individual %CV for the respective IgM autoantibodies levels (a 2.96–7.20 range). Acceptable range for inter-assay CV for an ELISA was determined to be below <10%.

IgM levels	Mean values (n=4)			Plate
	A450 WT	A450 SCR	A ₄₅₀ WT-SCR	
Threshold	0.262	0.191	0.071	1
Threshold	0.269	0.193	0.076	2
Threshold	0.277	0.206	0.071	3
Threshold	0.276	0.207	0.069	4
Low	0.375	0.192	0.184	1
Low	0.353	0.194	0.159	2
Low	0.357	0.170	0.187	3
Low	0.383	0.200	0.183	4
Medium	0.423	0.197	0.227	1
Medium	0.452	0.221	0.231	2
Medium	0.455	0.207	0.248	3
Medium	0.437	0.195	0.242	4
High	0.644	0.207	0.437	1
High	0.630	0.198	0.432	2
High	0.623	0.163	0.460	3
High	0.622	0.189	0.433	4

	Threshold	Low	Medium	High
Mean of Means	0.072	0.178	0.237	0.440
SD of Means	0.003	0.013	0.010	0.013
% CV of Means	3.97	7.20	4.19	2.96

Inter-assay CV = average % CV, 4.58