Multiple sclerosis (MS) is characterized by inflammation within the CNS. This inflammatory response is associated with production of nitric oxide (NO) and NO-related species that nitrosylate thiols. We postulated that MS patients would exhibit an antibody (Ab) response directed against proteins containing S-nitrosocysteine (SNO-cysteine) and showed that anti-NOCysteine Abs of the IgM isotype are in fact present in the sera of some MS patients (Boullerne et al., 1995). We report here the presence of a seemingly identical Ab response directed against SNO-cysteine in an acute model of MS, experimental autoimmune encephalomyelitis (EAE) induced in Lewis rats with the 68–84 peptide of guinea pig myelin basic protein (MBP68–84). Serum levels of anti-SNO-cysteine Abs peaked 1 week before the onset of clinical signs and well before the appearance of anti-MBP68–84 Abs. The anti-SNO-cysteine Ab peak titer correlated with the extent of subsequent CNS demyelination, suggesting a link between Ab level and CNS lesion formation. In relapsing–remitting MS patients, we found elevated anti-SNO-cysteine Ab at times of relapse and normal values in most patients judged to be in remission. Two-thirds of patients with secondary progressive MS had elevated anti-SNO-cysteine Ab levels, including those receiving interferon β-1b. The data show that a rise in circulating anti-SNO-cysteine Ab levels precedes onset of EAE. Anti-SNO-cysteine Abs are also elevated at times of MS attacks and in progressive disease, suggesting a possible role for these Abs, measurable in blood, as a biological marker for clinical activity.

Key words: experimental autoimmune encephalomyelitis; multiple sclerosis; autoimmunity; nitric oxide; antibody; clinical marker


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Anti-S-Nitrosocysteine Antibodies Are a Predictive Marker for Demyelination in Experimental Autoimmune Encephalomyelitis: Implications for Multiple Sclerosis

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Multiple sclerosis (MS), the most common inflammatory demyelinating disease of the CNS, usually begins as a relapsing–remitting process. As one attack follows another, disability accumulates, and usually the illness eventually becomes relentlessly progressive (Noseworthy, 1999). An easily measured biological marker that predicts MS activity would be desirable. None is known, except possibly soluble adhesion molecules (Rieckmann et al., 1998; Trojano et al., 1998), caspase mRNA expression (Furlan et al., 1999), and T-cell activation markers (Khouri et al., 2000).

Nonspecific inflammatory reactants, proposed as contributors to tissue damage in MS, include nitric oxide (NO). NO triggers axon and oligodendrocyte degeneration; both are targets in MS lesions (Boullerne et al., 2000; Smith et al., 2001). NO is volatile, so its production is usually measured indirectly by evaluation of NO synthases or NO-derived products such as nitrosated amino acids, nitrite, and nitrate. Increased nitrite level in CSF of MS patients correlates with clinical activity (Brundin et al., 1999; Svennensson et al., 1999). In active MS lesions, microglia and macrophages express inducible NO synthase (iNOS; Hooper et al., 1997), and macrophages isolated from an active MS lesion were shown to produce NO (De Groot et al., 1997). Similarly, blood monocytes produce substantial amounts of NO when MS is active (Sarchielli et al., 1997). In experimental autoimmune encephalomyelitis (EAE), an animal model for MS, iNOS mRNA expression within the CNS is enhanced both at onset and peak of clinical signs, with levels returning to normal on remission (Koprowski et al., 1993; Okuda et al., 1995). All the above suggests a relationship between NO production and CNS inflammation.

NO covalently modifies proteins. For example, nitrotyrosine, generated when tyrosine is nitrated by NO-related species, has been detected in MS and EAE lesions (Hooper et al., 1997; Cross et al., 1998). Similarly, cysteine can be nitrosated by NO and NO-related species (Hess et al., 2001), and new epitopes of possible relevance to MS can be generated. We have shown previously that antibodies (Abs) reactive with nitroso-S-cysteine coupled to the protein carrier bovine serum albumin (BSA) are elevated in sera from MS patients compared with sera from healthy controls (Boullerne et al., 1995), suggesting that anti-SNO-cysteine Abs might constitute a biological marker in MS and possibly a predictor of onset and severity of EAE.

We coupled S-nitrosocysteine (SNO-cysteine) to BSA and
measured serum levels of anti-SNO-cysteine Ab over the course of actively induced EAE in Lewis rats. We show that an anti-SNO-cysteine-BSA Ab response of the M isotype peaks 6–7 d postimmunization (dpi), i.e., several days before EAE onset, and that titers at this early time point correlate with the extent of subsequent demyelination. We also show that levels of serum anti-SNO-cysteine-BSA Abs are elevated in MS at times of relapse but normal in most patients judged to be in remission. Two-thirds of patients with progressive MS have elevated anti-SNO-cysteine-BSA Abs, including patients receiving interferon-β-1b (IFNβ-1b). Anti-SNO-cysteine-BSA Abs may serve as biological markers for disease activity in MS.

MATERIALS AND METHODS

Induction and clinical evaluation of EAE. Female Lewis rats aged 6–10 weeks were obtained from Charles River France SA. Animal handling conformed to guidelines of the European Community by the United Kingdom. Each rat received 100 μg of the encephalitogenic 68–84 peptide of guinea pig myelin basic protein (MBP 68–84; Y68GSLPQKSQ76R79SQDEN84; Peninsula Laboratories, Belmont, CA) in 50 μl of PBS emulsified with 50 μl of Freund’s adjuvant (Difco; Sparks, MD). Dorsal root ganglia (DRG) were preincubated at a dilution of 1:500 with 10 M SNO-cysteine-BSA to inhibit IgM binding to SNO-cysteine-BSA adsorbed on SA. Animal handling conformed to guidelines of the European Community by the United Kingdom.

Detection of rat anti-myelin basic protein antibodies. Abs directed against MBP 68–84 were assayed as described above for anti-SNO-cysteine-BSA Abs but with plates coated with MBP 68–84 at 0.5 μg/ml and with rat sera diluted 1:100. Each serum was tested in duplicate in two independent experiments for Abs to SNO-cysteine-BSA and to MBP 68–84 peptide, and the four OD values were averaged to give a mean Ab titer. Within-plate OD variation was 5–6%; between-plate OD variation was ±26%.

Determination of demyelination. The extent of myelin depletion was determined in cerebellar and C1–C6 cervical spinal cord white matter at 35 dpi. Animals were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused intracardially with 100 ml containing 5 × 10 M H11003 UF/ml heparin, followed by 500 ml of 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4. Brain and spinal cord were removed and immersed in 4% paraformaldehyde for 24 hr at 4°C. C1–C6 cervical spinal cord and cerebellum blocks were washed in 0.1 M phosphate buffer for 2 hr at 4°C and then incubated in a 30% sucrose solution in 0.1 M phosphate buffer overnight at 4°C for cryoprotection. Samples were stored at −80°C until processing. Sagittal cerebellum sections of 30 μm were cut using a cryostat (HM500M; Microm). Cerebellum sections were immunostained for MBP to evaluate myelin depletion according to a procedure adapted from Vandenberg et al. (1986). Free-floating adjacent sections of cerebellum were incubated overnight with polyclonal rabbit Ab against MBP diluted at 1:20,000 (kindly provided by Dr. A. Fournier, Victor Segalen University). Sections were then incubated with a biotin-labeled goat anti-rabbit Ab diluted at 1:200 (Dako SA, Trappes, France). Sections were then incubated with the biotin-labeled peroxidase-conjugated streptavidin technique using 3,3′-diaminobenzidine as chromogen (avidin–biotin complex kit; Dako) and incubation for 10 min. Negative controls were performed by omitting the primary Ab and by using Abs unrelated to CNS antigens. The mean staining intensity per defined square surface was measured using a densitometer system (Samba 2640; Alkelat) and expressed in arbitrary units of OD. For each section, MBP labeling was measured in the intact and demyelinated granular lamina of the different lobes. Blank values were obtained from the molecular layer of the cerebellum, known to contain primarily unmyelinated fibers. The average value of MBP staining per animal was calculated by measuring the mean value of four sections within lobes 4, 6, and 8 and then subtracting the mean of blank values.

Eleven series of five transverse cervical spinal cord sections (C1–C6) of 30 μm were cut using a cryostat (HM500M). The first section of each series (thus 150 μm apart) was stained for myelin by Spielmeyer’s method as described previously (De Castro and Ramon y Cajal, 1933). Myelin appears black after staining by Spielmeyer’s method. Free-floating sections were incubated in 2.5% ammonium iron (III) sulfate aqueous solution, washed in water, and stained with hematoxylin. To evaluate the amount of myelin loss in white matter on stained sections, an image analysis system (NIH Image running on an Apple Macintosh computer) connected to a microscope (Optika) was used to measure the mean of blank values.

Determination of inflammation. From the same series of cervical spinal cord cross sections (C1–C6), two sections (each series again 150 μm apart) were stained with hematoxylin and eosin. Infiltrations were observed perivascularly, within the parenchyma and in the meninges. An inflammatory index was calculated by averaging the total number of infiltration sites per section. The size of individual infiltration sites was weighted by a factor of 1–3 to reflect the intensity of the pathology. The maximum number of infiltration sites was 5 per section. Five to 11 sections were analyzed for each of nine animals. The total length of cervical spinal cord examined was 600–900 μm. The percentage of myelin loss was calculated by dividing the lesion size into the total area of white matter for each section.

Detection of rat anti-myelin basic protein antibodies. Abs directed against MBP 68–84 were assayed as described above for anti-SNO-cysteine-BSA Abs.
logical deterioration that had been evolving for >6 months and were classified as treated with IFNg-1b (4) or untreated (5).

Control sera were from 5 French and 19 American healthy volunteers. Titers of human anti-SNO-cysteine-BSA IgM were determined blind according to a procedure described previously (Boullerne et al., 1995). The method was as outlined above for the EAE experiments, except that serum or plasma was diluted 1:300, and Ab was revealed with peroxidase-conjugated rabbit anti-human IgM (M-chain-specific; Dako, Carpinteria, CA) diluted at 1:10,000. The average OD of each healthy donor group was used to determine the relative OD of serum or plasma of each MS patient according to the following calculation: relative OD = ODsample − mean ODhealthy group/mean ODhealthy group.

To detect any difference in anti-SNO-cysteine-BSA Ab level between serum and plasma of the same individuals, several paired samples from MS patients and controls were tested within the same assay. No difference in relative OD was detected. For this reason, either serum or plasma titers were measured in individual subjects.

**Specificity of IgM to SNO-cysteine-BSA.** Specificity of IgM binding to immobilized SNO-cysteine-BSA was assessed by adding SNO-cysteine-BSA or competitors to the liquid phase and measuring binding inhibition. Six MS sera chosen for their high Ab titer were preincubated at dilutions of 1:300 and 1:450 with competitors at concentrations of 10−6, 10−5, and 10−4 M in buffer C. After centrifugation at 10,000 × g for 10 min, or at 12,000 × g for 13 min, supernatants were tested by ELISA with SNO-cysteine-BSA adsorbed on plates. Competitors tested included cysteine-BSA (a homolog of SNO-cysteine-BSA) and SNO-cysteine-BSA preincubated with 2 mM HgCl2 (Sigma) in 0.3 M Tris-HCl buffer, pH 7.4, for 4 hr at 4°C, according to a previously described method (Saville, 1958), to destroy nitrosylation. After centrifugation, the supernatant was recovered, assayed for protein content, and incubated with MS sera.

Additional competitors included BSA-g nitrosylated by incubation with NaNO2 (Sigma) in 0.5 M HCl (NO-BSA-g) as described for SNO-cysteine-BSA (Boullerne et al., 1995); cysteine-BSA incubated with 0.1 M H2O2 (Fisher Scientific, Pittsburgh, PA) at 37°C for 30 min; or cysteine-BSA adsorbed on plates. Competitors tested included cysteine-BSA (a homolog of SNO-cysteine-BSA) and SNO-cysteine-BSA preincubated with 2 mM HgCl2 (Sigma) in 0.3 M Tris-HCl buffer, pH 7.4, for 4 hr at 4°C, according to a previously described method (Saville, 1958), to destroy nitrosylation. After centrifugation, the supernatant was recovered, assayed for protein content, and incubated with MS sera.

**RESULTS**

**Circulating anti-SNO-cysteine-BSA antibodies in EAE**

In the first experiment, all rats developed EAE. The time course of the clinical scores showed a biphasic pattern (Fig. 1A). The mean onset of the first episode occurred at 12.6 dpi, although one rat showed extremely early clinical signs with a score of 3 at 6 dpi. Mean clinical signs peaked at 17 dpi, with an average clinical score of 2.0 ± 0.577 (mean ± SD). Recovery was almost complete at 23 dpi, with an average clinical score of 0.43 ± 0.79. A second clinical episode occurred in four of seven rats with a mean onset at 28 dpi. Relapses lasted for 1 week, followed by complete recovery. All seven rats were killed after a 6-month observation period. No CNS pathological analysis was performed.

A 10-fold increase above baseline in the IgM response to SNO-cysteine-BSA was observed at 7 dpi. The average OD of sera sampled at 7 dpi was 0.779 ± 0.163 (mean ± SEM; range, 0.332–1.296) compared with 0.081 ± 0.131 (range, 0.044–0.125; p < 0.001, post hoc analysis) on the day before immunization (Fig. 1B). The IgM response at 14, 21, and 30 d was twofold to threefold elevated compared with baseline. The 7 dpi value was significantly greater than at later time points (p < 0.001, post hoc analysis). Animals that relapsed showed a higher Ab titer at 7 dpi (1.024 ± 0.211, n = 4) than animals with a single episode (0.452 ± 0.070, n = 3) although no secondary rise in Ab response on day 21 preceded relapses.

The specificity of IgM binding to SNO-cysteine-BSA was demonstrated by an inhibition experiment using various concentrations of SNO-cysteine-BSA in the liquid phase. This showed displacement of Ab binding proportional to the amount of SNO-cysteine-BSA added (Fig. 1C).

The IgG response to the SNO-cysteine-BSA epitope, although
Figure 1. First series of experiments on young rats aged 6–7 weeks (n = 7). A, Biphasic pattern of clinical course of EAE in 6- to 7-week-old rats. Clinical scores were graded daily for the scores 0–3 over a 35 d follow-up. Daily average clinical scores are plotted (mean ± SD). B, Serum anti-SNO-cysteine-BSA IgM titers peak 1 week after immunization in rats induced for EAE. Values are expressed as mean OD ± SEM of Ab titers. Anti-SNO-cysteine-BSA IgM titers in sera from EAE rats (open bars) were significantly elevated at 7 dpi when compared with other time points (**p < 0.001). IgG titers (closed bars) rose less than IgM titers but were nonetheless elevated at 7 dpi compared with other time points (*p < 0.05). No rise of anti-SNO-cysteine-BSA IgM was observed in the control group immunized with CFA alone (shaded bars). The control group immunized with CFA alone showed neither clinical signs nor an Ab response to SNO-cysteine-BSA. Each plot is the mean OD ± SEM of two independent experiments each performed in duplicate. C, Serum Ab response against MBP68–84 peptide in rats with EAE. Values are expressed as mean OD ± SEM of Ab titers obtained from two independent analyses each in duplicate. Anti-MBP68–84 IgM titers (open bars) were significantly elevated at 14 and 21 dpi when compared with other time points (*p < 0.05). Anti-MBP68–84 IgG titers (closed bars) were significantly elevated at 21 and 30 dpi (**p < 0.05).

less dramatic than the IgM response, also peaked at 7 dpi (Fig. 1B). IgG titers were threefold elevated at 7 dpi (0.288 ± 0.073; range, 0.090–0.543) when compared with baseline (0.064 ± 0.017; range, 0.028–0.150; p < 0.05, post hoc analysis). IgG titers decayed subsequently. The control group immunized with CFA alone showed neither clinical signs nor an Ab response to SNO-cysteine-BSA (Fig. 1B).

**Anti-SNO-cysteine-BSA antibodies precede anti-MBP antibodies**

Serum levels of anti-MBP68–84 IgM were significantly increased at 14 and 21 dpi in the EAE group when compared with other time points (p < 0.05, post hoc analysis). Anti-MBP68–84 IgG levels were significantly increased at 21 and 30 dpi (p < 0.05, post hoc analysis), as illustrated in Figure 1D. No Ab response directed against MBP68–84 was observed in the control group immunized with CFA alone (data not shown).

**Clinical signs and anti-SNO-cysteine-BSA antibodies**

In the second EAE experiment, using rats aged 9–10 weeks, mean disease onset occurred at 15.7 dpi, 3 d later than in the first experiment. Disease was milder, as expected for older rats, with an average clinical score of 1.0 ± 1.0 (mean ± SD). There was no second episode (Fig. 2A). Again, serum levels of anti-SNO-cysteine-BSA IgM at 6 dpi in the EAE group (n = 9) were significantly greater than at baseline (average OD, 0.492 ± 0.052; range, 0.315–0.720; vs average OD before immunization, 0.087 ± 0.008; range, 0.060–0.130; p < 0.001, post hoc analysis). Values were lower than in the first experiment in which disease was more severe. As in the previous study, anti-SNO-cysteine IgM peaked 1 week after immunization (Fig. 2B).

Again, serum levels of anti-MBP68–84 IgG at 30 dpi in the EAE group (n = 9) were significantly greater than at baseline (average OD, 0.463 ± 0.083; range, 0.100–0.910; vs average OD before immunization, 0.088 ± 0.004; range, 0.070–0.105; p < 0.001, post hoc analysis) but were lower than in the first experiment in which disease was more severe (Fig. 2C).

To determine whether individual values for anti-SNO-cysteine-BSA or anti-MBP68–84 Ab titers correlated with subsequent clinical severity, we compared the cumulative scores of all rats (n = 16) from the two experiments with their Ab titers. There was no
There was no correlation between MBP$_{68-84}$ Ab titers at any time point and cumulative clinical severity scores.

Correlation among demyelination, inflammation, and anti-SNO-cysteine-BSA antibodies

The extent of demyelination at 35 dpi, evaluated in the cervical spinal cord by Spielmeyer staining in EAE rats of the second experiment, varied substantially from rat to rat (Fig. 3). A direct relationship was found between the level of anti-SNO-cysteine-BSA Abs at 6 dpi and the extent of demyelination in the spinal cord ($r = 0.93; p = 0.0007; n = 9$; Fig. 4A) and between the level of anti-SNO-cysteine-BSA Abs at 6 dpi and the extent of inflammation in the spinal cord ($r = 0.85; p = 0.006; n = 9$; Fig. 4B).

The extent of myelin depletion in the cerebellum, as evaluated by quantitative immunostaining for MBP, revealed a 28% decrease in the EAE group (8.04 ± 0.52, mean ± SEM; $n = 9$) compared with control rats (11.12 ± 1.28; $n = 5$; $p = 0.02, t$ test). A direct relationship was found between the level of anti-SNO-cysteine-BSA Abs at 6 dpi and subsequent MBP depletion in cerebellum ($r = -0.82; p = 0.011; n = 9$; Fig. 4C). There was a strong correlation between demyelination in spinal cord and in cerebellum in EAE rats ($r = -0.87; p = 0.0045; n = 9$; Fig. 4D).

No correlation was found between anti-MBP$_{68-84}$ Abs at any time point and CNS demyelination or inflammation at 35 dpi.

Anti-SNO-cysteine-BSA antibodies in MS

Ab titers directed against SNO-cysteine-BSA varied with clinical activity (Fig. 5). Ab titers were of IgM isotype as noted previously (Boullerne et al., 1995). Ab titers are graphed as relative OD to permit for pooling of experiments using different groups of healthy controls and MS patients. All eight samples from six MS patients in relapse (one patient was studied during three relapses) showed significantly elevated titers of anti-SNO-cysteine-BSA IgM (1.879 ± 0.285, mean ± SEM; $n = 8$), when compared with
healthy controls (0.00 ± 0.092; n = 19; p < 0.001) and with both MS groups in remission, one group treated with IFNβ-1b (0.139 ± 0.224; n = 7; p < 0.01; one patient studied twice was negative on both occasions) and the other group untreated (0.521 ± 0.238; n = 11; p < 0.05; one patient studied on four occasions was positive twice, and another was negative on two occasions).

Two-thirds of secondary progressive MS patient samples (SPMS) showed elevated titers of anti-SNO-cysteine-BSA IgM whether treated with IFNβ-1b (1.107 ± 0.396; n = 9) or untreated (1.847 ± 0.375; n = 14). These elevations were significant when compared with the control group (untreated SPMS, p < 0.001; IFNβ-treated SPMS, p < 0.05) and with the two groups in remission (untreated SPMS vs IFNβ-treated patients in remission, p < 0.001; untreated SPMS vs untreated patients in remission, p < 0.01). Four untreated SPMS patients were studied serially, one on five occasions (patient A), one on three occasions (patient B), and two others twice (patients C and D). Eleven of 14 samples (79%) were positive, including 5 of 5 samples of patient A, 3 of 3 samples of patient B, 1 of 2 samples of patient C, and 0 of 2 samples of patient D. Eight SPMS patients receiving IFNβ were studied; one patient was studied twice. Five of 9 samples (55%) were positive, including both from the patient studied twice.

Because a correlation was found in sera of MS patients (but not in healthy donors) between total IgM levels and anti-SNO-cysteine IgM titers in our previous study (Boullerne et al., 1995), we attempted to rule out a nonspecific increase in anti-SNO-cysteine Ab simply attributable to IgM hyperglobulinemia. We measured anti-SNO-cysteine Ab titers in IgM purified from sera of four MS patients in relapse and from four healthy donors, all at the same protein concentration. A marked increase of anti-SNO-cysteine-BSA Ab titer was found in IgM purified from MS sera compared with IgM purified from healthy donor sera (Fig. 6).

**Specificity of anti-SNO-cysteine-BSA antibodies in MS**

To characterize the specificity of circulating IgM toward the nitrosylated cysteine residue linked to the BSA carrier by a five-carbon chain (glutaraldehyde linking), binding inhibition experiments were performed. First, sera from three MS patients were incubated with the following competitors: SNO-cysteine-BSA, SNO-cysteine-BSA treated with HgCl2 to abolish nitrosylation, and cysteine-BSA (a homolog for SNO-cysteine-BSA). Binding of MS serum IgM to SNO-cysteine-BSA absorbed on plates was inhibited by 50% (IC50) after exposure to 4 × 10−7 m soluble SNO-cysteine-BSA (Fig. 7A). HgCl2-treated SNO-cysteine-BSA inhibited binding poorly, as did cysteine-BSA, showing that nitrosylation is essential for binding of IgM to SNO-cysteine-BSA.

Additional inhibition experiments were performed using sera from 3 additional MS patients and the following competitors: SNO-cysteine-BSA, cysteine-BSA treated with HCl1 (a control for acidic nitrosylation by NaNO2), cysteine-BSA treated with the NO donor SNAP, cysteine-BSA treated with the peroxynitrite donor SIN-1, cysteine-BSA treated with H2O2, and BSA-g n-
trosylated by NaNO₂ (NO-BSA-g). Again, the strongest avidity was observed for SNO-cysteine-BSA, with an IC₅₀ of 2 × 10⁻⁷ M (Fig. 7 B,C). The other competitors failed to inhibit binding at 50%, even at 10⁻⁵ M. The findings indicate a high degree of specific binding to SNO-cysteine-BSA for circulating IgM from MS patients.

One MS serum was blotted on the following nitrosylated molecules: SNO-cysteine-BSA, NO-BSA-g, DTT-treated SNO-cysteine-BSA, DTT-treated NO-BSA-g, and HgCl₂-treated SNO-cysteine-BSA. Western Blots were performed under low SDS conditions to preserve nitrosylation. Preferential binding to SNO-cysteine-BSA was observed (Fig. 8). Some staining was observed after treatment of SNO-cysteine-BSA with DTT (2 hr at 4°C), but only faint staining remained after treatment of SNO-cysteine-BSA with HgCl₂, the most efficient treatment for destroying nitrosylation. NO-BSA-g was not stained. Again, binding of serum IgM to nitrosylated cysteine residues linked to BSA with glutaraldehyde was highly selective.

**Stability of SNO-cysteine-BSA**

Because nitrosylated compounds are often labile, we examined the stability of nitrosylated SNO-cysteine-BSA kept at room temperature and exposed to light over 15 d. We used two methods to evaluate nitrosylation. We assessed nitrosylation directly by scanning a solution of SNO-cysteine-BSA (1.26 mg/ml in 0.1 PBS) from 240 to 500 nm and recording the OD at 400 nm (OD₄₀₀), the peak of absorbance of nitrosylation. A 35% decrease of OD₄₀₀ was observed after a 24 hr exposure to room temperature and light (OD decreased from 0.270 to 0.176). OD₄₀₀ decreased further to 0.135 over the following 14 d. After complete denitrosylation (accomplished by incubation with 10 mM DTT at room temperature for 3 d), OD₄₀₀ was at background values. The above indicates that there was an ~50% loss of detectable nitrosylation over 2 weeks at room temperature.

We also used the Griess assay to measure nitrite and nitrate release from the SNO-cysteine-BSA solution described above. An increase of released nitrite and nitrate was observed over a 13 d exposure to room temperature and to light, increasing from 30 µM at day 1 to 49 µM at day 13 (Fig. 9). In contrast, complete

**Figure 6.** Anti-SNO-cysteine-BSA IgM Abs are enriched in the IgM of MS patients. Immunoglobulin fractions were tested at a protein concentration of 25 µg/ml. Anti-SNO-cysteine-BSA IgM is elevated in total IgM purified from the serum of an MS patient during relapse on a gel filtration column (closed squares) compared with a healthy donor (open squares). The mean of two independent experiments in duplicate is plotted. MS and control sera representative of four MS and four healthy donor sera tested are shown.

**Figure 7.** MS sera are specific for the SNO-cysteine epitope. IgM binding to immobilized SNO-cysteine-BSA is inhibited by a competitor in the liquid phase in a dose-dependent manner. Binding inhibition is calculated by dividing OD of each competitor concentration by OD without competitor (OD₀). A, Specific binding of IgM to immobilized SNO-cysteine-BSA (closed squares) with IC₅₀ at 4 × 10⁻⁷ M. Absence of nitrosylation, i.e., with cysteine-BSA (open squares) or SNO-cysteine-BSA treated with HgCl₂ (closed circles), prevented IgM binding inhibition. Each plot is the average OD ± SD of three sera tested in two independent experiments, each performed in duplicate. B, Specific binding of IgM to immobilized SNO-cysteine-BSA (closed squares) with IC₅₀ at 2 × 10⁻⁸ M. Conformational modifications of the BSA carrier induced by acidic nitrosylation (NO-BSA-g; open circles) or cysteine-BSA treated with HCl1 (closed circles), prevented IgM binding inhibition. Each plot is the average OD ± SD of three other sera tested in two independent experiments, each performed in duplicate. C, Other oxidative modifications of cysteine-BSA induced by the peroxynitrite donor SIN-1 (closed diamonds) or by hydrogen peroxide (closed circles) did not inhibit IgM binding to immobilized SNO-cysteine-BSA (closed squares). Each plot is the average OD of one serum tested in two independent experiments, each performed in duplicate.
denitrosylation followed incubation with 10 mM DTT for 3 d at room temperature and resulted in release of 154 μM nitrite and nitrate, close to the maximal expected value of 159 μM. Treatment with 10 mM DTT for 2 hr resulted in only partial release of nitrite and nitrate, in keeping with the Ab binding observed in blotting experiments when SNO-cysteine-BSA was treated for 2 hr with 10 mM DTT at 4°C (Fig. 8). Collectively, these observations indicate that nitrosylation of SNO-cysteine-BSA is adequately conserved during the 1–2 d interval required to coat the ELISA plates and perform the assay.

Origin of anti-SNO-cysteine-BSA antibodies in MS

To assess the origin of anti-SNO-cysteine-BSA antibodies in MS, we tested pairs of CSF and sera from MS patients. Circulating anti-SNO-cysteine-BSA antibody titers were elevated in 10 of 25 sera. Anti-SNO-cysteine-BSA antibodies were detected in only one CSF. The OD in this sample CSF diluted 1:2 was 0.207, and in the serum diluted 1:400 it was 0.219. We conclude that anti-SNO-cysteine-BSA antibody does not originate in the CNS.

DISCUSSION

We report an Ab response against an uncommon epitope, SNO-cysteine-BSA, in sera from Lewis rats with actively induced EAE. A similar Ab response is identified in MS. Titers of IgM Abs to SNO-cysteine-BSA did not correlate with the severity of clinical disease in EAE but did correlate with the extent of demyelination measured after clinical recovery, suggesting a possible role for anti-SNO-cysteine-BSA Abs in the demyelinating process.

A concern related to the stability of SNO-cysteine-BSA. The compound was shown to be stable over time. Generation of SNO-cysteine-BSA introduces conformational changes to the carrier protein BSA; therefore, it became important to establish that the epitope recognized was indeed nitrosylated cysteine. During the first step of SNO-cysteine-BSA generation, cysteine is linked to BSA by glutaraldehyde. This step would be expected to cross-link BSA itself. During the second step, chemical nitrosylation of cysteine might additionally nitrosylate tyrosine residues of the BSA carrier, possibly creating nitrotyrosine. We excluded an Ab response against glutaraldehyde-treated BSA and against BSA nitrosylated directly. Western blots failed to show binding of IgM to the modified BSA carriers. These negative results were reinforced by the failure of glutaraldehyde-treated BSA and nitrosylated BSA to inhibit IgM binding to SNO-cysteine-BSA bound to ELISA plates.

Another concern was that the Ab response might have been directed against some other oxidation product derived from the linked cysteine. Inhibition experiments established that the IgM response was specific for SNO-cysteine-BSA. The above supports the idea that the epitope recognized on SNO-cysteine-BSA conjugates is the nitrosylated cysteine moiety.

Our finding of anti-SNO-cysteine Abs provides evidence for the formation in vivo of S-nitroso complexes and for the development of Abs directed against new epitopes generated by NO-related species-mediated nitrosylation. NO-related species-mediated new epitope formation accounts for the finding of nitrotyrosine on proteins in CNS lesions both in MS and in active EAE (Hooper et al., 1997; Cross et al., 1998). Unlike nitrotyrosine in the CNS, the anti-SNO-cysteine-BSA Abs documented herein in blood peaked well before the onset of clinical signs or CNS inflammation, suggesting a potential role for them as a predictor of subsequent disease activity.

The anti-SNO-cysteine Abs must be directed against a peripherally expressed epitope. We found IgM anti-SNO-cysteine Abs in the CSF in only 1 of 25 MS cases, arguing strongly against a CNS origin for these Abs, at least in humans. Levels of the NO-derived products nitrite and nitrate increase in the plasma within 24 hr of immunization with MBP in rats (Cowden et al., 1998; O’Brien et al., 1999), indicating brisk early peripheral NO production and by extension prompt epitope expression. In contrast, iNOS mRNA in the CNS was first detected 4 d before disease onset in rodents immunized with MBP (Koprowski et al., 1993; Okuda et al., 1995). The peripheral origin of anti-SNO-cysteine IgM Abs is further supported by their detection in sera of rats with adjuvant arthritis and no CNS inflammation, albeit at a lower intensity than in rats with EAE (A. I. Boullerne, unpublished observations). Adjuvant arthritis is preceded by early production of NO, raised plasma levels of nitrite and nitrate, and elevated iNOS expression in the spleen (Fletcher et al., 1998). All of these appear preclinically, just as in EAE.

A second component of the humoral immune response, driven...
by MBP itself, was observed. IgM Abs directed against the MBP_{68–84} peptide peaked 2–3 weeks after immunization, as found previously in rats immunized with MBP_{68–84} peptide or with MBP (Kibler et al., 1977; Fesel and Coutinho, 1998). Note that the anti-SNO-cysteine-BSA IgM response peaked well before the anti-MBP IgM response, suggesting that the former was a natural Ab response (Avrameas and Ternynck, 1993). An IgG response against SNO-cysteine-BSA also occurred conjointly with the IgM response but was less intense, as has been reported previously for natural auto-Ab (Matsiota-Bernard et al., 1993). The early conjoint appearance of anti-SNO-cysteine-BSA IgM and IgG is typical for a natural Ab response known to be both early and chiefly of the IgM isotype, with lesser IgG isotype representation. In mice immunized with bacteria, natural Abs to DNA and to tubulin peak well ahead of specific antibacterial Abs (Matsiota-Bernard et al., 1993), in keeping with the time courses of anti-SNO-cysteine-BSA Abs and anti-MBP Abs observed here.

Anti-MBP_{68–84} Abs did not correlate with cumulative clinical signs in this study, as in numerous previous studies in EAE. Anti-SNO-cysteine-BSA antibodies similarly did not correlate with cumulative clinical signs but did correlate with demyelination and inflammation.

The role of circulating anti-SNO-cysteine Abs in EAE remains unknown. This stated, the correlation of anti-SNO-cysteine Abs at day 6 with subsequent demyelination argues against an epiphenomenon. Demyelination occurs in the EAE model that we used for this study, as in numerous previous studies in EAE. Anti-SNO-cysteine-BSA antibodies similarly did not correlate with cumulative clinical signs but did correlate with demyelination and inflammation.


