

# Nitric Oxide Localized to Spinal Cords of Mice with Experimental Allergic Encephalomyelitis: An Electron Paramagnetic Resonance Study

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## Summary

Experimental allergic encephalomyelitis (EAE) is a demyelinating autoimmune disorder that can be induced in susceptible mice by T lymphocytes sensitized to central nervous system (CNS) myelin components and is a prime animal model for the human CNS demyelinating disorder, multiple sclerosis (MS). Although CNS inflammation in which T lymphocytes and activated macrophages are the predominant cell types is observed in mice with EAE and in humans with MS, the exact mechanisms underlying the CNS damage and demyelination are not understood. Nitric oxide (NO), a gaseous free radical, has recently been shown to be a cytolytic product of activated macrophages. Using electron paramagnetic resonance spectroscopy, the nitric oxide free radical complexed with iron-sulfur proteins has been identified in affected spinal cords of mice with EAE, concurrent with the diminution of iron-sulfur proteins. These results indicate NO may play a role in the disease process of EAE, and perhaps MS.

EAE is a cell-mediated autoimmune demyelinating disorder that can be induced in several species. The murine form of EAE induced by adoptive transfer of CD4<sup>+</sup> myelin basic protein-specific T lymphocytes is especially useful as a model due to its clinical and histologic similarities to the human central nervous system (CNS)<sup>1</sup> inflammatory demyelinating disease multiple sclerosis (MS) (1–3). Macrophages form a significant component of the inflammatory cells in lesions of acute and chronic EAE and MS, and are the effector cells of demyelination (4, 5). However, the pathogenetic mechanisms underlying myelin damage and the production of neurologic signs for both disorders are not known.

Nitric oxide (NO), a free radical generated from guanidino nitrogens of L-arginine (6) in a reaction catalyzed by nitric oxide synthase (NOS), is now considered to be a biologic mediator (7–9). Recently, it has been shown that activated macrophages (as well as other cell types) synthesize large amounts of NO via a distinct, inducible isoform of NOS (iNOS) (10). NO thus generated appears to be under immunologic control (11, 12) and to be cytotoxic (13). Furthermore, NO may be a mediator of immune function (14) and

play a role in some autoimmune disorders (15), including EAE (16). Since electron paramagnetic resonance (EPR) spectroscopy is the definitive method by which free radicals can be identified and quantified (17), the present study investigated the presence of NO or its paramagnetic products in CNS, blood, and organs of mice with EAE using EPR.

## Materials and Methods

**Induction of EAE.** Female SJL/J mice (The Jackson Laboratory, Bar Harbor, ME), 6–8 wk of age, were maintained in accordance with National Institutes of Health guidelines in microisolators. EAE was induced by a method that is routine in our laboratory (18). Briefly, myelin basic protein (MBP) was prepared from guinea pig spinal cords (Rockland Inc., Gilbertsville, PA) as described (19) and donor mice were immunized over the flanks with 0.4 mg MBP in CFA containing *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). 10 d later the draining lymph nodes were removed, made into a single-cell suspension, and cultured 3 d with 50 µg MBP/ml. After washing, 0.5–1 × 10<sup>8</sup> cells were injected intravenously into syngeneic recipients, which developed clinical relapsing and remitting EAE. Mice were scored from 0 to 5 (0, normal; 1, limp tail; 2, difficulty righting; 3, one limb plegic; 4, two limbs plegic; 5, three limbs plegic, moribund) every 1–2 d using a published scale (20).

**Preparation of Tissue Samples for EPR.** At the time of death, anesthetized mice were bled from the heart, then perfused through the left cardiac ventricle with cold, sterile PBS to remove blood

<sup>1</sup> Abbreviations used in this paper: CNS, central nervous system; EPR, electron paramagnetic resonance; MBP, myelin basic protein; MS, multiple sclerosis; NO, nitric oxide; NOS, nitric oxide synthase.

from tissues. Extreme care was taken to avoid tissue decay; the spinal cords were removed intact and immediately frozen on dry ice in <10 min after death. Spinal cord weights ranged from 0.11 to 0.15 g. Blood was introduced into a quartz tube and frozen on dry ice within 2–5 min. In some cases, spleens and livers were removed and frozen on dry ice within 5 min of death.

**Treatment of Spinal Cord Tissue with NO.** To characterize the EPR spectra of the nitrosyl iron-sulfur protein complexes, ~0.05 ml of deoxygenated acetonitrile (CH<sub>3</sub>CN) saturated with NO gas was mixed with spinal cords from naive SJL mice as a positive control. To do this, acetonitrile in a vial capped with a rubber septum was first bubbled with nitrogen gas for 5 min to remove any dissolved gases, especially O<sub>2</sub>. NO gas (Matheson Gas Products, Secaucus, NJ) was then bubbled through the liquid acetonitrile for 1–2 min, after which the saturated solution was applied to the spinal cords in the EPR sample tube. The sample was immediately frozen on dry ice after the mixing.

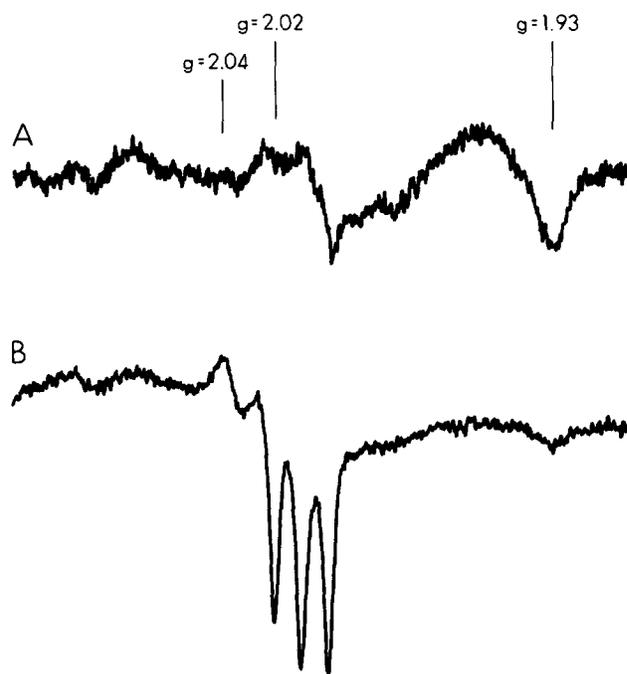
**Electron Paramagnetic Resonance.** EPR spectra were measured on spinal cords, the main area of pathology in our model (4), and blood from EAE-affected and healthy control SJL mice soon after death. Spleens and livers from two affected mice and two controls were also analyzed. An X-band spectrometer (ER200; Bruker, Billerica, MA) equipped with an EPR cryostat (4.2–300 K; Oxford Scientific, Concord, MA) was used. Signal averaging and data processing were implemented by an additional 12-bit analog/digital converter and a Scientific EPR software package. For each spectral analysis, two spinal cords were introduced into a 3–4-mm quartz tube (Wilmad Glass, Buena, NJ) and were stored at –70°C when not in use. To improve signal-to-noise, most of the present studies were performed within 3 h of death at cryogenic helium temperatures (10–20 K), with tissues being kept at –70°C during the intervening time. To compare the intensity of the peaks at  $g = 2.04$  from experiment to experiment, an “intensity index” defined as:  $10 \times$  (height of the peak at  $g = 2.04$ /the sum of the heights of the peaks at  $g = 2.04$  and 1.93) was determined for each pair of spinal cords. The peaks at  $g = 2.04$  and 1.93 represented nitrosyl iron-sulfur complexes and iron-sulfur protein, respectively (see below).

**Statistical Evaluation.** The Student's *t* test was used to compare the  $g = 2.04$  intensity indices of the EAE-affected and control spinal cord pairs.

## Results

**EPR Spectra of Normal Mouse Spinal Cords Exposed to NO Gas.** By EPR, NO has been shown to bind to iron-sulfur proteins resulting in an iron-nitrosyl complex with a characteristic  $g$  value of 2.04 (21–23). To confirm this with mouse spinal cords, EPR experiments were performed before and after a portion of deoxygenated acetonitrile saturated with NO was introduced to spinal cords taken from two naive mice. The results showed that NO induced a peak at  $g = 2.04$  that was not seen in the spectra of the untreated spinal cords (Fig. 1). Signal intensity at  $g = 2.02$  and 1.93 (iron-sulfur proteins; see below) noted in the untreated spinal cords were markedly reduced in the spectra of spinal cords exposed to NO (Fig. 1). The  $g = 2.04$  intensity index was 7.1 for the cord pair treated with NO gas. Residual blood in the spinal cords bound to excess NO to give a distinct NO-Fe heme protein signal at  $g = 2.01$  with a set of three <sup>14</sup>N hyperfine splittings (amplitude = 17 Gauss).

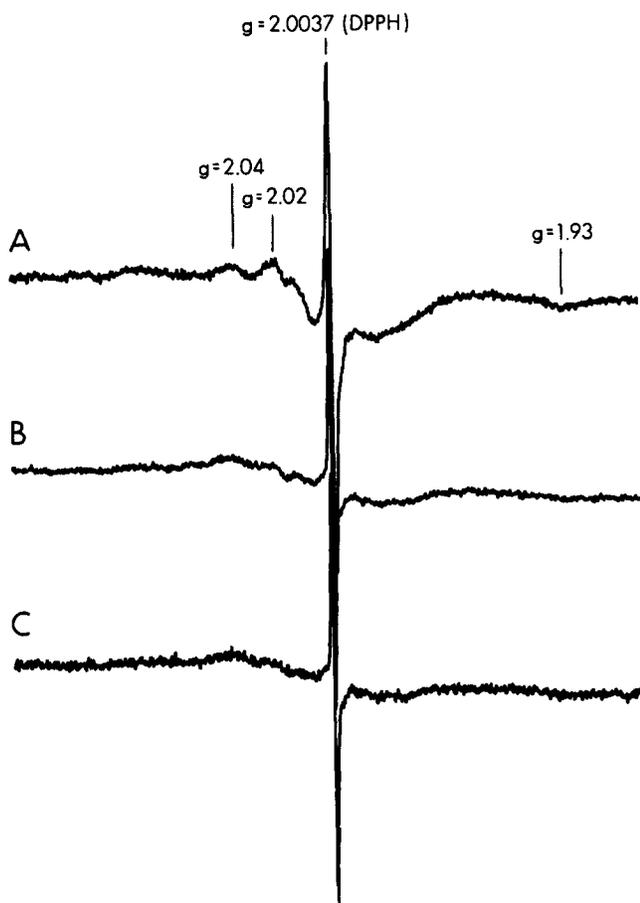
**Iron-Sulfur Proteins in Mouse Spinal Cords.** Oxidized iron-



**Figure 1.** EPR spectra of spinal cords from naive mice at 20 K: (A) before exposure to NO, (B) after exposure to NO dissolved in acetonitrile (positive control). Experimental settings: microwave frequency, 9.4437 GHz; modulation frequency, 100 KHz; modulation amplitude, 6.3 G; microwave power, 0.64 mW.

sulfur centers exhibit a fast-relaxing axial signal at  $g = 2.02$  at liquid helium temperatures that disappears when the temperature increases to 30 K (21). Another iron-sulfur signal occurs at  $g = 1.93$  (24). EPR spectra from 16 of 20 spinal cord pairs studied (32 of 40 EAE-affected and positive and negative control mice) were obtained at cryogenic helium temperatures (10–20 K). All 16 pairs exhibited signals at  $g = 2.02$  and 1.93, with the identical temperature dependence of iron-sulfur proteins (Fig. 2). The relative intensities of the iron-sulfur peaks ( $g = 1.93$ ) were more prominent in the spinal cord pairs from naive mice when compared with those of affected mice (Table 1).

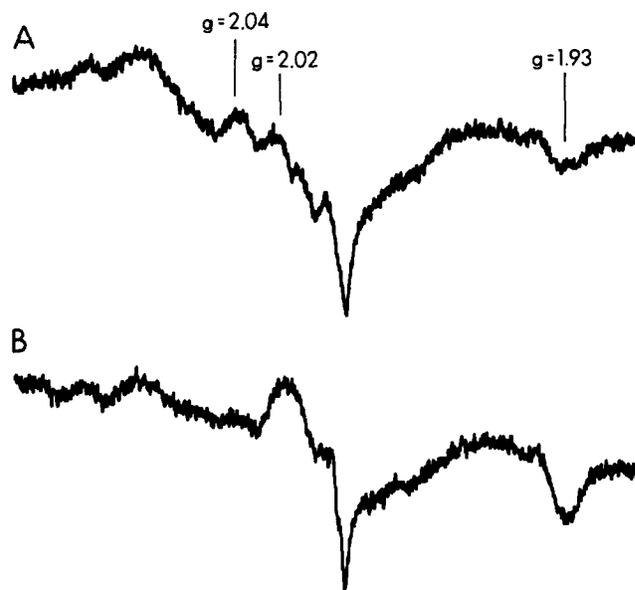
**Iron-Nitrosyl Complexes in Spinal Cords of Mice with EAE.** All 10 spinal cord pairs from SJL mice with EAE (clinical grades 1–4.5) exhibited EPR signals at  $g = 2.04$  (Table 1 and Fig. 3). In the initial EPR experiments, we analyzed three pairs of EAE-affected spinal cords at cryogenic nitrogen temperatures (–100°C), and all three showed a small peak at  $g = 2.04$  with signal-to-noise ratio ~2. Two pairs of the three were from acutely ill mice with scores of 2.5 and 4.0 in the first pair and both 4.0 in the second pair. The third pair derived from chronically ill mice with scores of 1.0 and 4.0. One pair of control unaffected cords analyzed at –100°C showed zero intensity at  $g = 2.04$ . To improve signal-to-noise, all subsequent spinal cord pairs were examined by EPR at cryogenic helium temperatures (10–20 K). The  $g = 2.04$  signal was further characteristic of iron-nitrosyl complexes (8, 24) in that it did not consist of a fast-relaxing component



**Figure 2.** EPR spectra of spinal cord of an EAE-affected SJL mouse at various temperatures: (A) 10, (B) 70, and (C) 100 K. Experimental settings: microwave frequency, 9.45 GHz; modulation frequency, 100 KHz; modulation amplitude, 5 G; microwave power, 1 mW. The stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as a  $g$  value marker ( $g = 2.0037$ ). Note that the signal-to-noise ratio for the peak at  $g = 2.04$  in A is higher than that in B or C.

(it could still be observed at 70 K in contrast to the signals at  $g = 2.02$  and 1.93 that disappear at temperatures  $>30$  K; see Fig. 2). The  $g = 2.04$  intensity index was significantly greater ( $p = 0.002$ ) for the spinal cord pairs from affected mice with EAE than for spinal cord pairs of naive mice (Table 1 and Fig. 3).

**EPR Measurements of Blood and Other Tissues.** To investigate whether NO production was increased systemically in mice with EAE, EPR measurements on individual samples of spleen and liver from two mice with EAE and two controls were performed. No EPR signals corresponding to iron-nitrosyl complexes were observed. Within blood samples, nitrosyl-heme proteins would be expected if NO were present (8). We did not observe nitrosyl-heme proteins ( $g = 2.012$  and 2.069) in 10 samples of blood from naive and EAE-affected mice that were frozen immediately after removal and analyzed by EPR within 4 h of death (data not shown). The absence of observable NO bound to Fe in heme proteins in blood or other organs may be attributable to the rapid reac-



**Figure 3.** EPR spectra of spinal cords of SJL/J mice at 10 K. (A) Two EAE-affected cords (clinical grades 1.5 and 3); (B) two naive mouse cords. Note the presence of a peak at  $g = 2.04$  in A and the reduction in the iron-sulfur protein peaks at  $g = 2.02$  and 1.93 in the affected (A) as compared with control (B) spinal cord. The broad background in A is due to residual heme. Experimental settings same as those of Fig. 2.

tion of NO with  $O_2$ ,  $O_2^-$ , and oxyhemoglobin to produce nitrate and methemoglobin (25). However, the  $g = 2.01$  NO-Fe heme protein signal with three  $^{14}N$  hyperfine splittings due to residual blood could be detected in some spinal cord pairs from mice with EAE.

## Discussion

In the present study, iron-nitrosyl complexes were identified by EPR spectroscopy in the target organ (spinal cord) of mice with the CNS autoimmune demyelinating disorder EAE. The complexes were significantly smaller in spinal cords of healthy control mice, indicating that the  $g = 2.04$  signal was not due to the small amount of NO known to be formed by endothelial cells and neurons (26) via the constitutive isoform of NOS (27). Iron-nitrosyl complexes were not observed in other organs of mice with EAE or control mice. The levels of iron-nitrosyl complexes observed in EAE-affected spinal cords were comparable to the high levels obtained when NO is synthesized via iNOS.

The reactivity of NO with other paramagnetic species such as iron ( $Fe^{2+}$ ) and oxygen is often deleterious to living cells (8). Much of the toxicity of NO is due to its high affinity for metalloproteins; release of NO with subsequent binding to and destruction of iron-sulfur centers (at near physiologic temperatures) causes tissue dysfunction via inhibition of the Krebs cycle, the electron transport chain, and DNA synthesis (28). Iron-sulfur centers, important to the function of a number of enzymes, can be identified by EPR (24). In cells treated

**Table 1.** Summary of EPR Findings on Spinal Cords at Cryogenic Helium Temperatures

Mice	Exp. and Run*	Mouse	Clinical grade†	DPT‡	Intensity index at $g = 2.04$ §
EAE	1	69/72	1/4	46/46	5.0
	2A	65/74	1.5/3	59/59	3.8
	3A	109/112	1/1.5	15/15	1.8
	4	110/113	4/4	15/15	2.6
	5A	76/97	1.5/3	75/48	2.2
	6A	139/140	4.5/4	28/28	2.5
	7A	260/262	1/4	14/14	4.4
Control	2B	114/115	Naive/naive	-/-	1.2
	3B	116/117	Naive/naive	-/-	1.4
	5B	59¶/62¶	0/0	94/94	1.2
	6B	155/156	Naive/naive	-/-	0.0
	7B	275/276	Naive/naive	-/-	1.4
	8	170/171	Naive/naive	-/-	0.5
	9	216/217	Naive/naive	-/-	0.6
	10	218/219	Naive/naive	-/-	0.4

Spinal cords were analyzed in groups of two for EPR measurement.

\* Subsets A and B of the same numeral refer to EAE-affected and control mice, respectively, killed and analyzed by EPR on the same day under identical EPR settings.

† Clinical grade 0–5 (see Materials and Methods).

‡ DPT, days post-cell transfer.

§ Intensity Index as described in Materials and Methods.

¶ Never clinically ill.

with NO, the iron-sulfur signal is known to decrease, with concurrent appearance of iron-nitrosyl signal (21), and in the present study when normal spinal cords were treated with NO, iron-sulfur signals became greatly diminished. In the affected spinal cords of mice with EAE, the ratio of iron-sulfur to iron-nitrosyl peaks was decreased in comparison with that in naive controls. This indicated that iron-sulfur proteins had converted to iron-nitrosyl complexes by reacting with NO.

The present study demonstrated NO production largely confined to the target organ in EAE, the CNS. The lack of strict correlation of the magnitude of the iron-nitrosyl complex with clinical scores remains unexplained, but may reflect sampling errors (e.g., involvement of the brainstem or cerebellum, which were not studied), the imprecise nature of the clinical scoring system, or the involvement of multiple factors in addition to NO in determining disease manifestations. No EPR signals corresponding to nitrosyl-heme proteins within the peripheral blood could be identified. Although hepatocytes are known to be induced by cytokines (IFN- $\gamma$ , TNF) to produce NO (29), an iron-nitrosyl signal was not observed in the liver, an organ unaffected by EAE. Perhaps any NO released from the CNS reacted locally with oxygen or superoxides before binding to heme proteins in the blood or distal organs.

The mechanisms leading to tissue damage in EAE and MS remain enigmatic. Though CD4<sup>+</sup> myelin-specific T lymphocytes are known to be the cells inducing EAE, most of the inflammatory cells within acute EAE lesions are nonspecific (30, 31), and include CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages, B lymphocytes, and neutrophils (4). In addition, other cells might be the source of increased NO production. In the rat model of EAE, NADPH diaphorase staining (known to recognize NOS) localized to the vasculature, suggesting upregulation of NOS in vascular or perivascular cells (R. G. Tilton, unpublished observations). Activated macrophages play a crucial role in EAE (32) and are particularly numerous in chronic, active lesions (33). Furthermore, two cytokines, IFN- $\gamma$  and TNF, known to be important to the development of EAE (34, 35) and present in MS lesions (36, 37), upregulates iNOS (38).

The observation of NO products within spinal cords affected by EAE raises the possibility that some of the tissue dysfunction and destruction in the EAE model may be secondary to local NO production. Furthermore, the NO free radical in target tissues of EAE in association with a relative decrease in iron-sulfur proteins suggests a potential pathogenetic mechanism. Additional support for a pathogenic role for NO in EAE was obtained when mice induced to develop EAE and treated with the iNOS-inhibitor aminoguanidine (39, 40) dis-

played lower maximum critical scores than did control-treated mice (Cross, A. H., R. F. Lin, T. P. Misko, W. F. Hickey, J. L. Trotter, and R. G. Tilton, manuscript in preparation). Cytotoxic effects of NO might impair the ability of oligodendroglia to remyelinate axons and might be involved in

oligodendroglial cell loss from affected tissues (41). Similarly, the axonal destruction that occurs in EAE (42) might involve NO toxicity. The present findings extend understanding of the pathogenic processes in the EAE model and may provide insight for its human counterpart, MS.

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